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Transcriptomic Profiling MicroRNA and Non-Coding RNA from Whole Blood in African Americans with MASLD

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Abstract: Metabolic dysfunction-associated steatotic liver disease (MASLD) is a growing health concern, yet the role of non-coding RNAs (ncRNAs), including microRNAs (miRNAs), in its pathogenesis remains poorly understood. In this pilot study, we aimed to identify significantly expressed miRNAs and ncRNAs and correlate transcriptomic patterns of the findings with previously identified coding gene expression profiles to explore potential regulatory mechanisms in MASLD**.** Participants were selected from an existing study population. We conducted transcriptomic profiling of miRNAs and other ncRNAs in whole blood samples from African American individuals with MASLD and matched controls (n=4 in each group). miRNA sequencing was performed by Zymo, USA followed by miRNA extraction using Zymo-Seq™ miRNA Library Kit. Differentially expressed RNAs were analyzed using Ingenuity Pathway Analysis (IPA) to identify associated biological pathways. A total of 1,412 miRNAs and 5,423 other ncRNAs were identified in MASLD patients compared to controls. Among them, 35 miRNAs and 28 other ncRNAs exhibited significant differential expressions (fold change cut off 1.5, $p < 0.05$). miR-206 was upregulated, potentially compensating for insulin resistance, while miR-1343-5p miR-1299, miR-224-5p, and miR-193a-5p were downregulated, connecting impaired lipid metabolism and fibrosis. IPA results identified hepatic fibrosis and cirrhosis pathways enriched with miRNA- other ncRNA interactions. Our findings highlight promising candidates for future biomarker validation and therapeutic targeting. Further large-scale studies are necessary to validate these candidates and elucidate their role in MASLD pathogenesis and ethnic disparities.

Keywords: microRNAs (miRNAs); non-coding RNAs (ncRNAs); MASLD; African Americans; transcriptomics

1. Introduction

Metabolic dysfunction-Associated Steatotic Liver Disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD), is recognized as the most prevalent chronic liver disease worldwide, affecting approximately 25–30% of the global adult population [1]. MASLD encompasses a spectrum of liver disorders, ranging from simple hepatic steatosis to non-alcoholic steatohepatitis

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(NASH), progressive fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2]. MASLD is strongly associated with metabolic syndrome, obesity, insulin resistance, type 2 diabetes mellitus (T2DM), and dyslipidemia, making it a major contributor to liver-related morbidity and mortality [3]. Despite its high prevalence, MASLD remains underdiagnosed and poorly understood in diverse ethnic populations, limiting the development of targeted therapeutic strategies [4].

A limited, but increasing, number of studies have underscored the regulatory roles of non-coding RNAs, particularly microRNAs (miRNAs), and other non-coding RNAs (ncRNAs) in the development and progression of MASLD [10,11]. The miRNAs are small non-coding RNA molecules that post-transcriptionally regulate gene expression by targeting mRNAs for degradation or translational repression. Small, non-coding RNA molecules also play a crucial role in regulating gene expression by targeting mRNA transcripts [39] by binding to the 3′ untranslated region (3′UTR) of target mRNAs, leading to mRNA degradation or inhibition of translation. This post-transcriptional regulation of gene expression helps control various cellular processes, including cell growth, development, and differentiation [39]. miRNAs play crucial roles both within cells and in the extracellular environment by regulating gene expression intracellularly and functioning as intercellular messengers when secreted into extracellular fluids. They are transported via exosomes or RNA-binding protein complexes such as those involving AGO proteins to modulate gene expression in target cells [40]. Previous studies have reported the relationship of multiple miRNAs in hepatic lipid metabolism, insulin signaling, inflammation, and fibrosis [12,13]. Similarly, other ncRNAs have emerged as critical modulators of hepatic gene expression, influencing hepatocyte apoptosis, inflammation, and fibrosis [14]. However, there have been few attempts to coordinate expression patterns of non-coding RNAs with the altered gene expression patterns in MASLD subjects, especially within the same individuals.

To determine the feasibility of addressing this issue, a case-control pilot study of the expression patterns of ncRNAs was conducted using individuals from a previous [7,8] transcriptomic investigation conducted by our group. This population was comprised of African Americans (AA) to address the lack of inclusion of minority participants in prior studies of transcriptomic patterns in MASLD patients. This study utilized whole blood samples which we and others have demonstrated to have substantial utility in establishing transcriptomic patterns that largely overlap patterns observed in hepatic tissue [7,8]. The current pilot study, to the best of our knowledge, reports for the first time the transcriptomic profiling study of miRNA and other ncRNA expressions in whole blood samples from AA individuals with MASLD.

2. Results

2.1. Study Participants

All selected participants (total n=8) were AAs and were part of the previous transcriptomic study from the Washington DC area [7]. The participants were separated into two groups: a control group of individuals without MASLD, and a case group of individuals with early stage MASLD. MASLD was diagnosed based on standard criteria including confirmed hepatic steatosis (based on their imaging/biopsy records supported by the presence of hepatic steatosis on cross-sectional imaging, liver elastography, and/or histological confirmation by percutaneous liver biopsy) and exhibited one or more comorbid metabolic features, viz., type 2 diabetes, hypertension, hyperlipidemia, or obesity [7]. Table 1 displays the characteristics of each group. No significant differences in age, BMI, and Hba1c were observed. To select the participants, we checked the data of BMI, HbA1c value, dyslipidemia, and presence of steatosis (as measured by FibroScan). For the purposes of liver steatosis staging, we used the S grade (S0 - S3);the higher the grade, the higher percentage of liver affected by fatty changes.

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Table 1. Participants Characteristics.

	Control (n=4)	MASLD (n=4)	P-value
Age (years)	61±4.83	53±5.94	0.08
Male/Female	2/2	2/2	-
BMI (kg/m^2)	25.22±1.53	27.8±4.25	0.29
Hba1c (%)	5.26±0.37	5.65±0.07	0.27
LDL (Optimal range <100 mg/dL)	-	137.34±26.65	-
HDL (Optimal range 40-70 mg/dL)	-	50.34±18.82	-
Triglyceride (Optimal range <150 mg/dL)	-	124±85.08	-
FibroScan*	-	Patient 1- F0; Patient 2- F2-F3; Patient 3- F0-F1; Patient 4- F0-F1	-
Steatosis Stage**		Patient 1- S3; Patient 2- S3; Patient 3- S1; Patient 4- S3	-

*F0-Normal, F1-Mild fibrosis, F3-Moderate fibrosis, and F4-Severe fibrosis. **S0- steatosis less than 11% (normal), S1- steatosis 11% to 33%, S2- steatosis 34% to 66%, S3- steatosis greater than 67%.

2.2. miRNA and Noncoding-RNA Sequencing, Differential Expression, Number of Reported Studies

Out of a total of 1412 miRNA identified transcripts, 35 miRNAs in the MASLD cases were significantly differentially expressed when compared to the controls (fold change cutoff 1.5-fold and p-value < 0.05) with 24 downregulated and 11 upregulated (**Figure 1A** and **Table 2**). Out of a total of 5423 other ncRNAs transcripts, 28 were significantly differentially expressed with 17 downregulated and 11 upregulated in the MASLD cases compared to the controls (**Figure 1B** and **Table 3**).

We also examined the differential miRNA expression of each individual case compared to the control group's expression status (**Figure 2A**). We observed miR-1299, miR-193a-5p, miR-185-3p, miR-3960, miR-1343-5p, and miR-224-5p, were significantly downregulated and miR-206 significantly upregulated in all the MASLD subjects.

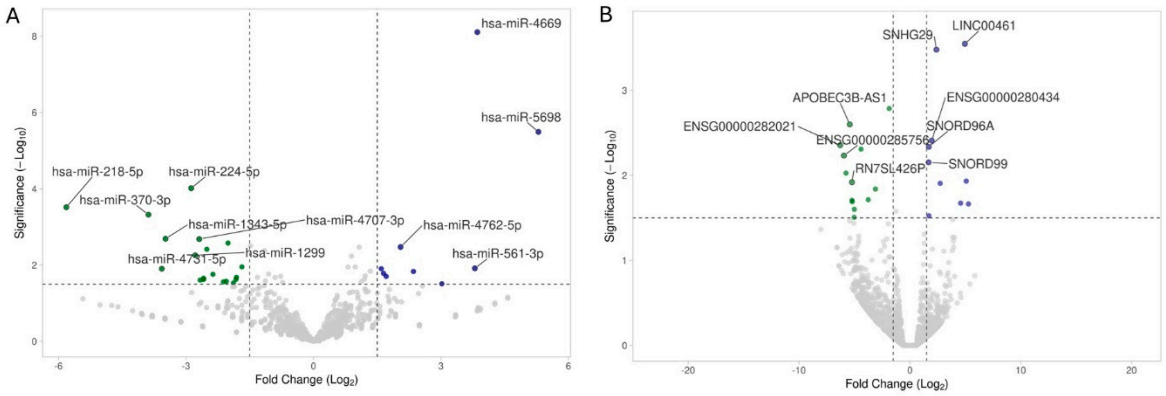

Figure 1. Transcriptomic Profiling miRNA and other ncRNAs from whole blood. (A) Volcano plot showing differential expression micro-RNA (log2 of fold-change; x axis) and statistical significance of this change (log10 of significance; y axis) in comparison of MASLD cases compared to the control group. (B) Volcano plot showing differential expression other ncRNAs (log2 of fold-change; x axis) and statistical significance of this change (log10 of significance; y axis). Colored points represent differentially expressed miRNAs and other ncRNAs (cutoff FDR 0.05) with magnitude of change 1.5 that are either overexpressed (**blue**) or under expressed (**green**). Most significant are labeled.

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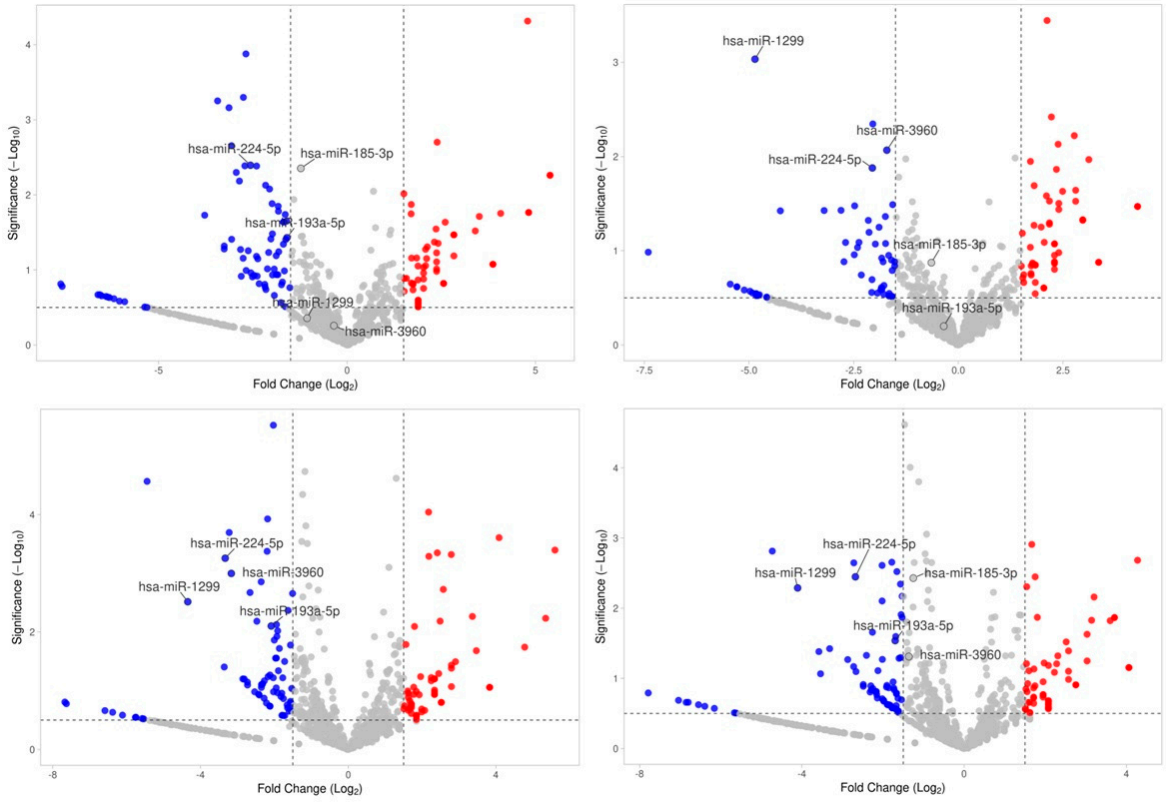

Figure 2. Volcano plot showing differential expression miRNAs (log2 of fold-change; x-axis) and statistical significance of this change (log10 of significance; y-axis) in comparison of each MASLD case compared to the control group. Colored points represent differentially expressed miRNAs (cutoff FDR 0.05) with magnitude of change 1.5 that are either overexpressed (red) or under expressed (blue). Common miRNAs are labeled.

Table 2. Differentially expressed miRNAs in MASLD subjects.

miRNA	Fold Change	P-Value	Biological Functions
hsa-miR-218-5p	-5.82	0.0003	Regulates placental development, airway inflammation, and hepatic lipogenesis; targets TGFβ2, SMAD2, TLR4, Elovl5. [41]
hsa-miR-370-3p	-3.88	0.0005	Regulates VSMC phenotype, glioblastoma suppression, and sinus node dysfunction in heart failure. [42]
hsa-miR-4731-5p	-3.57	0.0125	Tumor suppressor in glioblastoma, melanoma, and NSCLC; impacts viability, EMT, and apoptosis. [43]
hsa-miR-1343-5p	-3.48	0.0020	Reduces TGF-β signaling and fibrosis via exosomal delivery; therapeutic potential in lung disease. [28]
hsa-miR-224-5p	-2.88	0.0001	Promotes EMT in hepatocellular carcinoma, regulates autophagy in breast cancer, and modulates cardiovascular inflammation. [44]
hsa-miR-193a-5p	-2.80	0.0031	Tumor suppressor; inhibits proliferation and metastasis in ovarian and prostate cancers. [45]
hsa-miR-1299	-2.78	0.0055	Tumor suppressor; inhibits NEK2 in prostate cancer, also regulates RHOT1 and PDL1 in other cancers. [46]

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hsa-miR-4707-3p	-2.69	0.0021	Modulates cell fate in human neocortex development. [47]
hsa-miR-133a-3p	-2.67	0.0247	Tumor suppressor in colorectal cancer; inhibits angiogenesis. [48]
hsa-miR-365a-3p	-2.59	0.0236	Promotes lung cancer via PI3K/AKT; affects osteogenesis by targeting RUNX2. [49]
hsa-miR-4664-5p	-2.59	0.0223	Detected in breast cancer; potential cancer biomarker. [50]
hsa-miR-539-5p	-2.51	0.0039	Inhibits pancreatic cancer proliferation; regulates Tregs in leukemia. [51]
hsa-miR-369-5p	-2.37	0.0175	Inhibits hepatocellular carcinoma by targeting HOXA13. [52]
hsa-miR-150-3p	-2.12	0.0275	Antitumor in lung cancer; enhances neuronal proliferation. [53]
hsa-miR-1185-1-3p	-2.05	0.0267	Biomarker for weight loss response; associated with lung cancer. [54]
hsa-miR-3940-3p	-2.01	0.0026	Promotes granulosa cell proliferation; linked to insulin resistance in pregnancy. [55]
hsa-miR-369-3p	-1.90	0.0373	Anti-inflammatory; inhibits preadipocyte proliferation and differentiation. [56]
hsa-miR-452-5p	-1.89	0.0297	Regulates fibrosis and promotes cancer progression. [57]
hsa-miR-323b-3p	-1.86	0.0363	Upregulated in Huntington's disease; involved in neurodegeneration. [58]
hsa-miR-433-3p	-1.82	0.0236	Suppresses glioma growth; enhances chemotherapy sensitivity. [59]
hsa-miR-379-5p	-1.81	0.0209	Plays a role in regulating cellular processes, particularly in cancer development and progression. [60]
hsa-miR-409-5p	-1.71	0.0480	Promotes tumor growth, EMT, and bone metastasis in prostate cancer. [61]
hsa-miR-487b-3p	-1.69	0.0112	Negative regulator of skeletal myogenesis; suppresses C2C12 myoblast proliferation. [62]
hsa-miR-154-5p	-1.65	0.0451	Triggers cardiac oxidative stress and inflammation; tumor suppressor in glioblastoma. [63]
hsa-miR-3195	1.60	0.0125	Suppresses osteosarcoma progression by targeting SOX4; linked to prostate cancer. [64]
hsa-miR-6758-5p	1.65	0.0165	Specific function remains unknown.
hsa-miR-4479	1.7	0.0198	Potential biomarker in cancer; roles in immunosuppression and metastasis. [65]
hsa-miR-196a-5p	1.7	0.0437	Oncogene; promotes invasion, metastasis, and proliferation in many cancers. [66]
hsa-miR-4762-5p	2.0	0.0034	Detected in breast cancer tissues; role in tumorigenesis is under study. [67]
hsa-miR-129-5p	2.35	0.0147	Tumor suppressor; inhibits proliferation in hepatocellular carcinoma. [68]
hsa-miR-206	2.56	0.0353	Involved in cancers, neurodegenerative, and cardiovascular diseases; tumor suppressor. [69]
hsa-miR-4645-5p	3.02	0.0309	Facilitates diabetic wound healing by restoring keratinocyte autophagy. [70]

 doi:10.20944/preprints202506.0499.v1

hsa-miR-561-3p	3.80	0.0122	Modulates CX3CL1 signaling in hepatocellular carcinoma; suppresses metastasis. [71]
hsa-miR-4669	3.85	<0.0001	Enhances tumor aggressiveness; creates immunosuppressive environment in liver cancer. [72]
hsa-miR-5698	5.29	<0.0001	Identified as breast cancer biomarker; functions not well characterized. [73]

The above list of miRNAs includes those that are differentially expressed in the MASLD group (n=4) compared to the control group (n=4), with a fold change cutoff of ±1.5 (or at least 1.5-fold) and a p-value < 0.05.

Table 3. Differentially expressed other ncRNAs compared to controls.

Other ncRNA	Fold Change	P-Value	Biological Functions
Homo_sapiens_tRNA-Leu-AAG-1	-8.03	0.043	Encodes a tRNA specific for leucine with the AAG anticodon, essential for protein synthesis.
ENSG00000282021	-6.29	0.004	Specific function remains unknown.
ENSG00000285756	-5.95	0.006	Specific function remains unknown.
DLX6-AS1	-5.76	0.009	Long non-coding RNA implicated in promoting tumor cell proliferation, migration, invasion, and epithelial-mesenchymal transition in various cancers. [74]
FMNL1-DT	-5.44	0.034	Specific function remains unknown.
APOBEC3B-AS1	-5.42	0.003	Specific function remains unknown.
RN7SL426P	-5.23	0.012	Specific function remains unknown.
ENSG00000254639	-5.23	0.020	Specific function remains unknown.
RSF1-IT1	-5.20	0.020	Specific function remains unknown.
ENSG00000273064	-5.07	0.036	Specific function remains unknown.
PRDM16-DT	-5.03	0.031	Long non-coding RNA involved in regulating astrocyte function and implicated in colorectal cancer metastasis and drug resistance. [75]
RNU6-70P	-5.02	0.025	Specific function remains unknown.
Homo_sapiens_tRNA-Gly-GCC-5	-4.41	0.005	Encodes a tRNA specific for glycine with the GCC anticodon, essential for protein synthesis.
U8	-3.75	0.019	Specific function remains unknown.
NFE4	-3.11	0.014	Transcription factor involved in regulating fetal γ-globin gene expression. Acetylation of NFE4 prevents its ubiquitination and modulates its interaction with histone deacetylase HDAC1, influencing gene activation. [76]
Homo_sapiens_tRNA-Met-CAT-6	-1.95	0.037	Encodes transfer RNA for methionine with anticodon CAT, essential for initiating protein synthesis.
Homo_sapiens_tRNA-Asp-GTC-2	-1.86	0.002	Encodes transfer RNA for aspartic acid with anticodon GTC, facilitating incorporation of aspartic acid during protein synthesis.
SNORD99	1.69	0.007	Small nucleolar RNA involved in 2′-O-methylation of ribosomal RNA. Overexpression promotes endometrial cancer development by inhibiting GSDMD-mediated pyroptosis. [77]
SNORD96A	1.71	0.005	Small nucleolar RNA implicated in ribosomal RNA modification. Elevated levels in plasma

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			serve as a non-invasive diagnostic biomarker for clear cell renal cell carcinoma (ccRCC). [78]
SNORD48	1.71	0.030	Small nucleolar RNA involved in post-transcriptional modification of other small nuclear RNAs. Associated with prostate and hematologic cancers. [79]
ENSG00000280434	1.97	0.004	Specific function remains unknown.
SNHG29	2.40	0.000	Long non-coding RNA that regulates cell senescence via p53/p21 signaling and promotes glioblastoma progression through the miR-223-3p/CTNND1 axis. [80]
LINC01138	2.74	0.012	Long intergenic non-coding RNA that acts as an oncogenic driver by interacting with PRMT5, enhancing its stability, and promoting tumorigenicity in hepatocellular carcinoma. [81]
ENSG00000253374	3.86	0.033	Specific function remains unknown.
RN7SL33P	4.58	0.021	Specific function remains unknown.
LINC00461	4.96	0.000	Long non-coding RNA important for glioma progression, affecting cell proliferation, migration, and invasion via MAPK/ERK and PI3K/AKT signaling pathways. [82]
ENSG00000286834	5.09	0.012	Specific function remains unknown.
WDFY3-AS2	5.28	0.022	Long non-coding RNA that acts as a tumor suppressor by inhibiting cell proliferation and metastasis through the Wnt/β-catenin signaling pathway in oral squamous cell carcinoma. [83]

The above list of ncRNAs includes those that are differentially expressed in the MASLD group (n=4) compared to the control group (n=4), with a fold change cutoff of ±1.5 (or at least 1.5-fold) and a p-value < 0.05.

Table 4. Micro-RNAs differentially expressed in all MASLD subjects.

miRNA ID	Fold Change at p-Value <0.05	Role in MASLD
miR-206	2.22±0.19	miR-206 regulates lipid metabolism and fibrosis in MASLD by downregulating FGF21 and modulating the MAPK pathway.
miR-1343-5p	-3.98±2.50	miR-1343-5p contributes to MASLD by modulating the PI3K/Akt pathway, promoting hepatic lipid accumulation and inflammation.
miR-224-5p	-2.65±0.52	miR-224-5p exacerbates MASLD by activating the TGF-β/Smad pathway, promoting liver fibrosis and inflammation.
miR-1299	-3.59±1.71	miR-1299 plays a role in MASLD by inhibiting the Wnt/β-catenin pathway, thereby reducing hepatic fibrosis and lipid accumulation.
miR-193a-5p	-1.79±0.26	miR-193a-5p contributes to MASLD by deactivating the JNK/c-Jun pathway, which reduces inflammation and hepatic injury.
miR-185-3p	-2.59±1.06	miR-185-3p mitigates MASLD by inhibiting the NF-κB pathway, reducing inflammation and liver damage.

 doi:10.20944/preprints202506.0499.v1

miR-3960	-1.64±0.95	miR-3960 contributes to MASLD by activating the SIRT1/AMPK pathway, promoting lipid metabolism and reducing hepatic steatosis.

3.4. Top Biofunctions, Canonical Pathways, and Network Analysis

The IPA analysis revealed significant biofunctions, including "*fibrosis of liver*" and "*cirrhosis of liver*," with significant overlap percentage (p-value <0.05). These biofunctions were associated with 10 distinct miRNAs: miR-100-5p, miR-1273h-5p, miR-130a-3p, miR-133a-3p, miR-135a-5p, miR-143-3p, miR-16-5p, miR-199a-5p, miR-27a-3p, and miR-526a-5p (**Figure 2A, 2B**).

In addition to the miRNA findings, other ncRNAs were prominently featured in the network analysis (**Figure 3**). Several long non-coding RNAs (lncRNAs), such as *LINC00963*, *SNHG7*, *CYTOR*, and *HORMAD2-AS1*, were identified as key regulators in the hepatic fibrosis and cirrhosis pathways. These lncRNAs interacted with critical molecular hubs, including *EZH2*, *AKT*, and *YAP1*, highlighting their regulatory roles in fibrosis-related processes. The network further identifies ncRNAs, such as *RP11* and *LINC01234*, contribut to the modulation of gene expression, emphasizing their potential involvement in liver disease pathogenesis. The canonical pathway analysis (**Figure 4**) highlighted the "hepatic fibrosis signaling pathway" as a central mechanism linking these ncRNAs and miRNAs to critical molecular and cellular functions. The interactions between miRNAs, lncRNAs, and target genes suggest a tightly regulated network underlying MASLD.

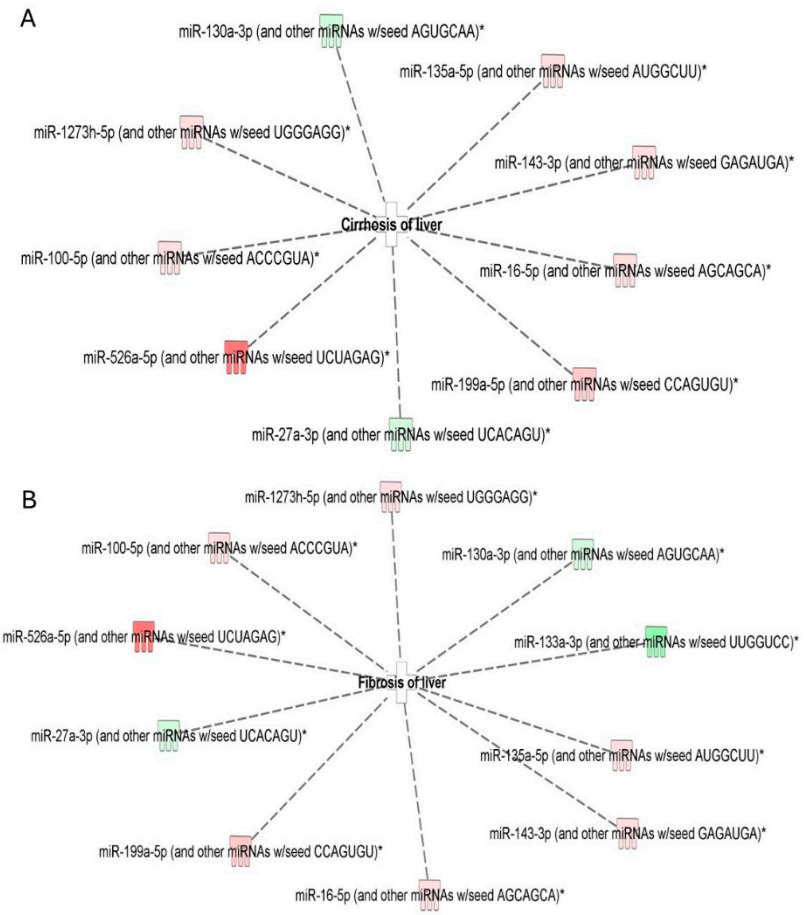

Figure 3. Ingenuity Pathway Analysis (IPA)-identified the number of differentially expressed miRNA connected to fibrosis and cirrhosis of the liver. (**A**) Differentially expressed miRNAs connected with fibrosis. (**B**) Differentially expressed miRNA connected with cirrhosis.

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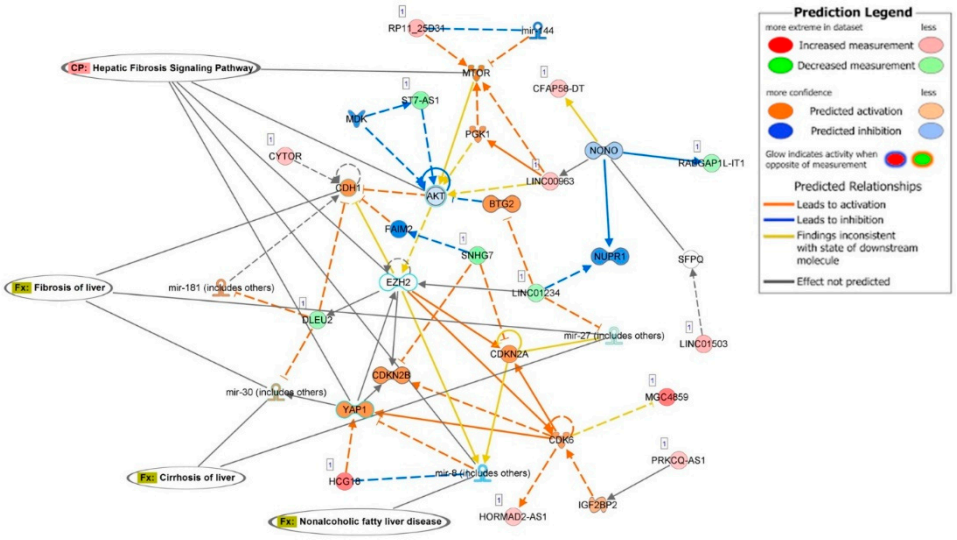

Figure 4. Network of differentially expressed ($p<0.05$) other ncRNAs data in the MASLD participants, relative to control group. The network was generated using Ingenuity Pathway Analysis (IPA) from QIAGEN, USA.

3. Discussion

In this pilot study, we present the first transcriptomic analysis of miRNA and other ncRNA expression profiles in whole blood samples from AA individuals with MASLD. By identifying differentially expressed ncRNA species, our work aims to generate hypotheses on regulatory pathways involved in MASLD development.

We observed that, among the most commonly dysregulated miRNAs , miR-206, miR-1299, miR-224-5p, and miR-193a-5p emerged as key candidates modulating hepatic lipid metabolism, insulin signaling, and fibrogenesis. miR-206, which plays a dual role in lipid metabolism and insulin sensitivity, has been shown to inhibit hepatic lipogenesis and gluconeogenesis, thereby promoting insulin responsiveness [23]. Consistent with these findings, our data reveal a 2.5-fold upregulation of miR-206 in all four MASLD patients, suggesting a protective response to hepatic insulin resistance [24].

miR-224-5p is known to be involved in multiple regulatory processes such as lipid accumulation, endoplasmic reticulum stress, mitochondrial damage, inflammatory response, autophagy, and hepatic stellate cell activation, potentially influencing the progression of MASLD [20,25]. Multiple studies have reported an upregulation trend of miR-224-5p, in serum, plasma, or liver biopsy samples [18–21]. Upregulated miR-224-5p targets the leptin (LEP) gene and leads to its suppression through dysregulation of the AMPK pathway, which is associated with MASLD progression [18]. Interestingly, in the current study we observed a significant downregulation of miR-224-5p in all four cases, with an average -2.65-fold change. These findings are consistent with the observation that AAs experience a slower progression of MASLD to cirrhosis and liver-related events compared to other racial groups [26].

miR-1343-5p is known to act as a potent repressor of TGF-β signaling and fibrosis through the direct attenuation of both canonical TGF-β receptors [28]. The downregulation of miR-1343-5p in all four MASLD patients in our study supports our previously published findings, which noted an upregulation of TGF-β and activation of the hepatic fibrosis signaling pathway in these subjects [7]. Another previous study reported that upregulation of circulating miR-1343-5p is a potential biomarker in MASLD in adolescents with severe obesity, [27]. Although our study included only adults' patients with MASLD, it is well reported that the dysregulation of TGF-β (Transforming Growth Factor-beta) signaling is associated with obesity and its related metabolic disorders [84].

Notably, the significant downregulation of miR-193a-5p in the current study, which has been previously reported as a biomarker for liver fibrosis and cirrhosis [29–34], reinforces its potential role

 doi:10.20944/preprints202506.0499.v1

as an early indicator of MASLD. The downregulation of miR-193a-5p is thought to inhibit pro-fibrotic gene targets, thereby promoting hepatic stellate cell activation, a central event in the fibrotic cascade [35]. Our prior research identified dysregulation of the *TGFB1* and *E2F1* genes and associated pathways in peripheral blood samples in the cohort of AA patients with early-stage MASLD [7] used for the current study. We observed the activation of hepatic fibrosis signaling pathways and their potential role in the development of hepatocellular carcinoma, particularly when *TGFB1* was upregulated and *E2F1* was downregulated [7]. However, the study did not establish a definitive role for *TGFB1* and *E2F1* regulation in the development of hepatic steatosis or the lower prevalence of MASLD in AAs. Some previous studies have reported an upregulation trend of miR-193a-5p in blood serum and plasma samples, mainly in Caucasian populations [29–31].

In addition to miRNAs, our pathway and network analyses identified several long non-coding RNAs (lncRNAs) involved in fibrosis-related signaling networks. LncRNAs such as LINC00963, SNHG7, CYTOR, and HORMAD2-AS1 interact with pivotal regulatory molecules including EZH2, AKT, and YAP1. EZH2, a histone methyltransferase, has been implicated in the progression of fibrosis, while YAP1, a core component of the Hippo signaling pathway, plays a critical role in liver regeneration and fibrogenesis [37]. Our canonical pathway analysis indicates strong associations between several ncRNAs (LINC00963, HCG18, ST7-AS1, RP11_25D31, CYTOR and LINC01234) and hepatic fibrosis pathways. The notable upregulation of WDFY3-AS2 and LINC02767 suggests that these lncRNAs may contribute to hepatic inflammation and fibrotic remodeling, although further validation is required in larger populations [38].

Although our study offers new hypotheses on the transcriptomic landscape of MASLD in African Americans and demonstrates the utility of whole blood samples for such investigations, it also has some limitations. The small study size restricts the generalizability of our findings and is vulnerable to selection bias. We focused on patients with early stage MASLD, for whom future interventions might prevent the progression of the disease. Additionally, we were unable to segregate our data to analyze other ncRNAs individually for each patient, as the data was analyzed and provided in group-wise format by the manufacturer of the analysis tools used, which needs to be addressed for future investigations. Future studies should incorporate larger, multi-ethnic cohorts and additional analytical tools to enhance statistical power and validate these preliminary observations. As our analysis was based on whole blood RNA profiles, the findings may not fully recapitulate hepatic transcriptomic alterations. Future research therefore should attempt to include matched analyses of liver tissue with the circulating plasma miRNAs to provide a more comprehensive understanding of MASLD pathogenesis.

4. Materials and Methods

4.1. Study Particepants and Blood Sample Collection.

In this pilot study, the study participants consisted of eight individuals (control n=4; MASLD n=4), with equal numbers of males and females, who self-identified as AA and were born in the USA. All participants responded to an advertisement through Howard University and Georgetown University Community Newsletter via email and/or flyers and public announcements and were recruited with their informed consent. The protocol was approved by Georgetown-MedStar IRB (MODCR00002260). Participants with MASLD were recruited from the MedStar Georgetown Transplant Institute. We selected only those patients who were at early stages of development MASLD (confirmed hepatic steatosis and exhibited one or more comorbid metabolic features, viz., type 2 diabetes, hypertension, hyperlipidemia, or obesity). Individuals with progression to severe fibrosis or cirrhosis were not included because of the small size of the participant groups and the wide spectrum of tissue features present during different stages of liver disease; we chose to limit subject inclusion to earlier stages of liver disease to increase the homogeneity of disease presentation in the different subjects. Individuals with severe fibrosis or cirrhosis were not included; we chose to limit subject inclusion to earlier stages of liver disease to increase the homogeneity of disease

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presentation in the different subjects (Table 1). Patients with other potential causes of liver disease, including viral, immunological, iron storage disease, Wilson disease, or alpha 1 antitrypsin deficiency, were excluded from the study. Participants with heavy alcohol use were also excluded from the study. Control participants were those who responded to the same flyers and advertisements described above, but did not have MASLD. These individuals were negative for self-reported HCV and HBV and had normal liver enzyme profiles. A questionnaire was provided to all participants to collect demographic and clinical information.

4.2. RNA Extraction and miRNA Library Preparation

Whole blood was collected in a DNA/RNA Shield™ Blood Collection Tube (Manufacturer: Zymo Research, Cat # R1150) during recruitment by experienced phlebotomists. Blood collection tubes were prefilled with 6 ml DNA/RNA Shield™ for direct collection of up to 3 ml whole human blood. DNA/RNA Shield lyses cells, inactivate nucleases and infectious agents (e.g., viruses and pathogens), and is ideal for safe sample storage and transport at ambient temperatures. RNA was extracted from DNA/RNA Shield tubes using the Quick-DNA/RNA™ Blood Tube Kit (Zymo Research, Cat. # R1151) according to the manufacturer's instructions. DNA contamination was removed using an Applied Biosystems Inc. (ABI) DNA-free kit (ThermoFisher, CA, Cat # AM 1906). RNA was quantified using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA and RNA.

We used Zymo-Seq™ miRNA Library Kit (Catalog Numbers: R3006, R3007) to generate small RNA libraries. To acquire miRNAs, present in total RNA or cell-free RNA extracted from biofluids we followed manufacturer's instructions and protocol. Briefly the protocol was with five major steps, which are adapter ligation and blocking, circularization and dimer removal, reverse transcription, index PCR, and library purification. At the time of index PCR pre-mixed forward and reverse primers with the following sequence:

Forward Primer Sequence:

5′-AATGATACGGCGACCACCGAGATCTACACNNNNNNNN(NN)ACAC TCTTTCCCTACACGACGCTCTTCCGATCT-3′

Reverse Primer Sequence:

5′-CAAGCAGAAGACGGCATACGAGATNNNNNNNN(NN)GTGACTGGA GTTCCTTGGCACCCGAGAATTCCA-3′

4.3. Sequencing and Data Analysis

The Sequencing was performed by Zymo Research (Irvine, California, US). Data analysis was performed according to instruction mentioned in the Zymo-Seq™ miRNA Library Kit (Catalog Numbers: R3006, R3007). To read the Zymo-Seq™ miRNA libraries we used bioinformatics tools (QIAGEN CLC Genomics Workbench) designed for Illumina's TruSeq Small RNA libraries. Prior to sequence alignment, sequenced reads processed with adapter trimming. For the trimming we used sequence of TGGAATTCTCGGGTGCCAAGG. In the final analysis data was extracted in the form of fold change and p-values keeping false discovery rate (FDR) at the level of $p<0.05$.

4.4. Ingenuity Pathway Analysis (IPA)

IPA was utilized to explore complex biofunctions within a biological system, identifying functional roles, molecular processes, and key networks associated with significantly differentially expressed genes in participants with MASLD. From the differential expression of miRNAs and other ncRNAs datasets described above, the identification of cellular processes and pathways by IPA (Qiagen, USA) was performed according to the methods described in our earlier study [15–17]. Briefly, datasets comprising miRNAs and other ncRNAs identifiers and corresponding expression values (fold-change) from the sequencing data were imported into IPA. Differentially expressed

 doi:10.20944/preprints202506.0499.v1

identifiers (miRNAs and other ncRNAs) were mapped to related changes in biofunctions [16]. The networks were generated algorithmically based on their connectivity. Using IPA, we identified the top network by amalgamating a large set of differentially expressed miRNAs and other ncRNAs with the goal of uncovering the most extensive array of relationships among the focus genes [13]. A score (P-score = − log10 (p-value)) according to the fit of the set of supplied genes and a list of biological functions stored in the Ingenuity Knowledge Base are generated [15] Networks were "named" on the most prevalent functional group(s) present. Canonical pathway analysis identified function-specific genes that were significantly present within the networks.

5. Conclusions

Our study is among the first to generate hypotheses on the significance of miRNA and ncRNA expression patterns in MASLD among AAs. The pathway analyses reveal correlations between these ncRNA patterns and the transcriptomic patterns of coding genes, particularly in pathways involving TGFB1 and E2F signaling. The findings also suggest that miR-206 upregulation may represent a protective response to insulin resistance. miR-206, miR-370-3p, and miR-193a-5p downregulation could contribute to MASLD pathogenesis via impaired lipid metabolism and fibrosis promotion. Other ncRNAs such as LINC00963, SNHG7, and CYTOR are implicated in hepatic fibrosis signaling. These findings highlight promising candidates for future biomarker investigation and therapeutic targeting. Further large-scale studies, including longitudinal transcriptomic profiling, will be essential to elucidate the role of ncRNAs in MASLD progression and ethnic disparities.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Georgetown-MedStar IRB (protocol code MODCR00002260).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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