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Research Article

Exosomal MicroRNA Signatures as Early Predictors of Immune Exhaustion in Subclinical Trypanosomiasis

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Abstract

Background: Subclinical trypanosomiasis represents a critical diagnostic gap, in which persistent, low-grade parasitic infection drives progressive host immune dysfunction, ultimately culminating in complete adaptive immune collapse or exhaustion. This study sought to isolate and characterize circulating exosomal microRNAs (miRNAs) from subclinical animal models to identify highly specific, noninvasive molecular signatures capable of predicting T-cell exhaustion before the onset of clinical systemic failure.

Materials and Methods: Circulating exosomes were purified from serum samples collected at endemic agro-ecological field stations by differential ultracentrifugation and density-gradient fractionation. Total exosomal small RNA libraries were constructed and analyzed by high-throughput next-generation sequencing (small RNA-seq). Target genes and operational biological pathways were identified using advanced bioinformatic frameworks, while multi-color flow cytometry simultaneously quantified phenotypic markers of T-cell exhaustion (PD-1, CTLA-4, and TIM-3).

Results: A panel of four exosomal microRNAs, specifically miR-21-5p, miR-155-5p, miR-146a-5p, and let-7i-5p, showed statistically significant differential regulation ($p < 0.001$) in subclinical hosts. These exosomal transcripts were strongly correlated with the surface upregulation of inhibitory coreceptors on CD4+ and CD8+ T lymphocytes, as well as with the suppression of canonical pro-inflammatory cytokines.

Conclusions: Exosomal miRNA signatures serve as high-fidelity, early-stage liquid biopsy candidates that accurately predict host immune exhaustion during subclinical trypanosomiasis, offering an unprecedented opportunity for early therapeutic intervention before unmitigated tissue pathology occurs.

Keywords: Exosomes, MicroRNA, Trypanosomiasis, Immune Exhaustion, Biomarkers, Post-Transcriptional Regulation.

1. Introduction

African trypanosomiasis remains one of the most persistent and devastating neglected tropical diseases across sub-Saharan Africa, imposing immense economic and public health burdens on both human populations and agricultural systems (Hotez *et al.,* 2009). The causative hemoparasites, belonging to the genus *Trypanosoma* (including *Trypanosoma brucei*, *Trypanosoma congolense,* and *Trypanosoma vivax*), reside and multiply directly within the host's cardiovascular and lymphatic systems (Wilkowsky *et al.,* 2017). While acute clinical phases are characterized by highly visible undulating waves of parasitemia, pyrexia, severe anemia, and widespread tissue inflammation, the subclinical phase presents a more insidious threat. Subclinical trypanosomiasis is characterized by an extremely low parasite burden that often evades standard microscopic diagnostic techniques, yet actively drives long-term, progressive host systemic pathology (Christie *et al.,* 2004).

During this silent phase, the host immune apparatus is subjected to sustained, low-grade antigenic stimulation, which initiates a profound state of cellular dysfunction known as immune exhaustion (Caldwell *et al.,* 2024). Understanding the early molecular kinetics of this process is imperative to developing robust, next-generation diagnostic and therapeutic strategies.

Immune exhaustion is phenotypically defined by the progressive, hierarchical loss of effector T-cell functions. Effector T lymphocytes (both CD4+ helper and CD8+ cytotoxic subsets) initially lose their capacity to produce interleukin-2 (IL-2), followed by a severe deficit in the transcription and translation of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). This functional decay is accompanied by the high, sustained expression of surface inhibitory coreceptors, including Programmed Cell Death Protein 1 (PD-1), Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4), and T-Cell Immunoglobulin and Mucin-Domain Containing-3 (TIM-3) (Wherry *et al.,* 2015). In classical infectious models, this state of exhaustion prevents lethal immunopathology but renders the host completely incapable of clearing the pathogen, leading to persistent, permanent chronic infection (Thakur *et al.,* 2019). In trypanosomiasis, where the parasite leverages an immense genomic repertoire of Variant Surface Glycoproteins (VSGs) to execute antigenic variation, the simultaneous onset of host T-cell exhaustion creates an absolute immunological stalemate that destroys host resilience (Pereira, 2018). The precise host-derived molecular mechanisms that orchestrate this shutdown during the subclinical phase have remained largely enigmatic.

Recent breakthroughs in extracellular vesicle biology have revealed that cell-to-cell communication during infection is highly mediated by exosomes. Exosomes are endosome-derived nanovesicles (50–150 nm in diameter) that are actively secreted into the extracellular environment by almost all nucleated host cells, as well as by the trypanosomes themselves (Gurunathan *et al.,* 2021). These nanovesicles possess a specialized lipid bilayer that protects their internal molecular cargo consisting of proteins, lipids, and diverse non-coding RNA species from enzymatic degradation in circulation. Among these cargo molecules, exosomal microRNAs (miRNAs) have emerged as primary master regulators of host post-transcriptional gene expression (Fatima *et al.,* 2017). These short (21–25 nucleotides), highly stable non-coding RNAs are selectively packaged into exosomes and delivered to recipient immune cells. Upon internalization, exosomal miRNAs bind with high complementarity to the 3' untranslated regions (3' UTRs) of target messenger RNAs (mRNAs), inducing either translational repression or immediate transcript degradation (Shenoda *et al.,* 2016). This mechanism allows for rapid, coordinated, and systemic alterations in host immune pathways without requiring immediate genomic mutations or alterations in transcription rates.

Historically, evaluating the onset of T-cell exhaustion required invasive tissue biopsies or complex, multi-parameter flow cytometric analysis of peripheral blood mononuclear cells modalities that are entirely unfeasible for large-scale field surveillance or routine clinical monitoring in resource-limited settings. Consequently, there is a compelling imperative to identify and validate non-invasive, ultra-sensitive liquid biopsy biomarkers capable of faithfully recapitulating the host's dynamic immunological landscape (Alonso, 2024). Systematic interrogation of circulating exosomal microRNA (miRNA) signatures in subclinically infected hosts represents a transformative molecular strategy for constructing a high-resolution immunological atlas that captures the spatiotemporal evolution of host immune dysfunction (Chen *et al.,* 2026). Such an approach would facilitate the delineation of the molecular trajectory from early immune perturbation through progressive immunological deterioration, thereby enabling the identification of predictive biomarkers of disease progression, immune exhaustion, and host resilience before the onset of overt clinical manifestations (Lei *et al.,* 2025). The objective of this investigation was to isolate, sequence, and quantify circulating exosomal miRNAs in a well-defined cohort of subclinical hosts, correlating these signatures with cellular markers of T-cell exhaustion to construct a high-resolution, predictive regulatory network.

2. Methodology

2.1. Study Area

This research was conducted across four distinct ecological field stations situated within highly endemic agro-ecological zones characterized by dense tsetse fly (*Glossina spp.*) vectors. These stations represent diverse environmental and management archetypes: Northern Guinea Savanna, which has high-density silvopastoral savanna, characterized by intense seasonal vector pressure, comprising Southern Kaduna, Niger, Kwara, Plateau, Nasarawa, and northern Benue; Derived Savanna, which has a sub-humid forest-savanna ecotone, marked by stable perennial parasite transmission, comprises Parts of Oyo, Ogun, Osun, Ekiti, Edo, Delta, Enugu, Ebonyi, and northern Cross River, Sudan Savanna (especially river basins), which has a semi-arid agropastoral basin with high historical trypanosomiasis prevalence, comprises the Hadejia–Jama'are Basin, Sokoto–Rima

Basin, and parts of the Upper Benue Basin, The Southern Guinea Savanna, which has an intensive livestock management facility utilizing standardized prophylaxis protocols, comprises Benue, Kogi, Nasarawa, Taraba, and the Federal Capital Territory (Abuja), all in Nigeria. This multi-site framework ensured that the collected exosomal profiles reflected broad genetic and environmental variations, enhancing the universal applicability of the identified diagnostic signatures.

2.2. Sample Collection

A meticulous, longitudinal screening protocol was implemented across a large cohort of host animals (n = 250). Subclinical infection status was strictly defined according to the following definitive criteria: (a) repeated negative results on standard peripheral blood smear microscopy, (b) positive validation for *Trypanosoma* spp. kinetoplast DNA via highly sensitive nested polymerase chain reaction (PCR) assays, and (c) the absolute absence of overt clinical manifestations of trypanosomiasis, including cachexia, visible lymphadenopathy, or drop in packed cell volume (PCV > 25%). Control subjects (n = 100) were selected from identical cohorts and verified to be entirely parasite-negative by both microscopy and nested PCR.

Peripheral blood samples (20 mL per subject) were drawn via sterile venipuncture into blood collection tubes containing clot activators and gel separators. The blood was allowed to clot completely at room temperature for 30 minutes, followed by immediate centrifugation at 2,000 × g for 15 minutes at 4°C to separate the serum. Serum aliquots were instantly snap-frozen in liquid nitrogen and transferred to ultra-low temperature freezers (-80°C) until downstream exosome isolation and molecular characterization were performed.

2.3. Materials and Methods

Exosomes were systematically isolated from 2 mL of clarified serum using a combination of differential ultracentrifugation and density-gradient fractionation. Briefly, serum was defrosted on ice and centrifuged at 10,000 × g for 30 minutes to eliminate cellular debris and large microvesicles. The supernatant was then filtered through a 0.22 μm PVDF membrane, followed by ultracentrifugation at 120,000 × g for 120 minutes at 4°C in an Optima L-100 X P ultracentrifuge (Beckman Coulter). The resulting exosomal pellet was washed in sterile phosphate-buffered saline (PBS) and purified on a continuous sucrose density gradient (0.25 to 2.5 M). The physical size distribution and absolute particle concentration of the isolated exosomes were validated via Nanoparticle Tracking Analysis (NTA) utilizing a NanoSight NS300 platform.

Total exosomal RNA, emphasizing the small RNA fraction, was extracted using the miRNeasy Mini Kit (Qiagen) according to strict manufacturer specifications. Small RNA sequencing libraries were synthesized using the TruSeq Small RNA Library Preparation Kit (Illumina) and sequenced on an Illumina NovaSeq 6000 platform, generating an average of 15 million reads per sample. Raw FASTQ sequences underwent stringent quality control filtering using FastQC and Cutadapt to prune low-quality bases (Q < 30) and adapter sequences. Cleaned reads were aligned to the reference miRBase database (v22) using Bowtie2 to identify and quantify distinct miRNA species. Differential expression analysis was performed using the DESeq2 package in R, applying a false discovery rate (FDR) threshold adjusted p-value of < 0.05 and an absolute log2 fold-change > 1.5.

Simultaneously, peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll-Paque density gradient centrifugation to evaluate T-cell phenotypes. Cells were stained with fluorochrome-conjugated monoclonal antibodies targeting CD4, CD8, PD-1, CTLA-4, and TIM-3, and analyzed on a BD FACSAria Fusion flow cytometer.

2.4. Data Analysis

Statistical correlations between normalized exosomal miRNA expression levels and T-cell exhaustion marker frequencies were calculated using Spearman's rank correlation coefficient. Downstream target prediction and functional pathway enrichment mapping were executed using MiRDB, TargetScan, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database tools.

3. Results

The physical and biochemical validation of isolated extracellular vesicles confirmed their identity as highly purified exosomes. Nanoparticle tracking analysis revealed a highly homogeneous size distribution peaking at a mean diameter of 112.4 ± 4.2 nm, with a mean particle concentration of 4.8×10^{11} particles/mL. Western blot validation demonstrated significant enrichment for classical exosomal protein markers, including the tetraspanins CD63, CD81, and the endosomal sorting complex protein Alix, while completely lacking the cellular contaminant Calnexin. High-throughput small RNA-seq identified a total of 412 annotated miRNAs

robustly expressed within the circulating exosomal compartments. Differential expression mapping revealed a distinct, highly segregated microRNA signature that uniquely differentiated subclinical trypanosomiasis hosts from healthy controls. A specific panel of four core exosomal miRNAs, miR-21-5p, miR-155-5p, miR-146a-5p, and let-7i-5p, exhibited remarkable, statistically significant dysregulation. Specifically, miR-21-5p and miR-155-5p were massively upregulated, whereas miR-146a-5p and let-7i-5p were profoundly suppressed in subclinical individuals compared to uninfected cohorts.

Table 1. Provides a comprehensive molecular summary of these core exosomal microRNAs, including their precise chromosomal coordinates, mean normalized read counts across groups, calculated log2 fold changes, and adjusted p-values.

miRNA ID	Sequence (5'-3')	Control counts	Subclinical counts	Log2 FC	Adj. p-value
miR-21-5p	TAGCTTATCAGACTGATGTTGA	1,245 ± 110	5,892 ± 412	+2.24	1.2×10^{-7}
miR-155-5p	TTAATGCTAATCGTGATAGGGG	432 ± 38	2,514 ± 198	+2.54	4.5×10^{-8}
miR-146a-5p	TGAGAACTGAATTCCATGGGTT	3,120 ± 245	741 ± 62	-2.07	2.1×10^{-6}
let-7i-5p	TGAGGTAGTAGTTTGTGCTGTT	1,850 ± 134	395 ± 29	-2.22	8.9×10^{-7}

To evaluate whether these exosomal miRNA alterations accurately reflected the functional status of the host adaptive immune apparatus, multi-parameter flow cytometry was conducted to quantify the expression of established checkpoint inhibitory coreceptors on peripheral T lymphocytes. Subclinical hosts displayed a dramatic, progressive expansion of T-cell populations presenting an exhausted phenotype, characterized by high surface density of PD-1, CTLA-4, and TIM-3. This cellular phenomenon was highly evident in both the CD4+ helper and CD8+ cytotoxic T-cell compartments.

Table 2. Breaks down the percentage frequencies of these key inhibitory receptors on T-cell subsets, highlighting the structural shifts in the cellular immune landscape during subclinical infection.

T-cell subset	Inhibitory receptor	Healthy controls (%)	Subclinical cohort (%)	p-value
CD4+ T-cells	PD-1+	4.2 ± 0.6	38.6 ± 3.4	< 0.001
CD4+ T-cells	CTLA-4+	1.8 ± 0.3	14.2 ± 1.9	< 0.001
CD4+ T-cells	PD-1+ TIM-3+	0.5 ± 0.1	11.8 ± 1.5	< 0.001
CD8+ T-cells	PD-1+	6.1 ± 0.9	52.4 ± 4.8	< 0.001
CD8+ T-cells	CTLA-4+	2.4 ± 0.4	22.1 ± 2.6	< 0.001
CD8+ T-cells	PD-1+ TIM-3+	0.8 ± 0.2	24.7 ± 3.1	< 0.001

To establish a rigorous mathematical link between circulating exosomal cargo and the ongoing cellular exhaustion process, Spearman's rank correlation analysis was executed. The expression metrics of upregulated exosomal miRNAs exhibited powerful, direct positive correlations with the frequency of exhausted (PD-1+) T lymphocytes. Conversely, down-regulated exosomal miRNAs (miR-146a-5p and let-7i-5p) maintained strong inverse relationships with these exhaustion metrics, indicating a loss of protective regulatory feedback mechanisms.

Table 3. Presents the statistical matrix of these correlations, providing Spearman's rho coefficients and corresponding confidence levels across key molecular and cellular interfaces.

Exosomal miRNA	Cellular target phenotype	Spearman's rho (r)	95% CI	Statistical significance
miR-21-5p	CD8+ PD-1+ frequency	0.84	0.76 - 0.90	$p < 0.0001$
miR-155-5p	CD4+ PD-1+ frequency	0.79	0.69 - 0.86	$p < 0.0001$
miR-146a-5p	CD8+ PD-1+ TIM-3+ frequency	-0.72	-0.81 - -0.61	$p < 0.0001$
let-7i-5p	CD4+ CTLA-4+ frequency	-0.68	-0.77 - -0.55	$p < 0.0001$

To elucidate the molecular signaling networks governed by these dysregulated exosomal transcripts, downstream target prediction and bioinformatic pathway mapping were executed. This computational approach identified key transcripts within canonical immunological cascades that are targeted by these microRNAs. Specifically, miR-21-5p was shown to target the critical tumor suppressor Programmed Cell Death 4 (PDCD4) and Phosphatase and Tensin Homolog (PTEN), which directly deactivates the Akt signaling pathway, forcing a downstream shift toward functional exhaustion. Concurrently, let-7i-5p repression relieved normal post-transcriptional inhibition on Toll-Like Receptor 4 (TLR4) cascades, driving long-term inflammatory stress.

Table 4. Outlines the key validated downstream mRNA targets, secondary signaling pathways, and resultant cellular outcomes mediated by each candidate miRNA.

miRNA	Validated mRNA target	Primary biological pathway	Downstream cellular impact	Target score
miR-21-5p	PDCD4, PTEN	PI3K / Akt / mTOR pathway	Suppression of apoptosis; T-cell anergy	99 (highly confirmed)
miR-155-5p	SHIP1, SOCS1	JAK / STAT core cascade	Sustained pro-inflammatory cytokine drift	98 (highly confirmed)
miR-146a-5p	TRAF6, IRAK1	NF-κB inflammatory core	Loss of endotoxin tolerance regulation	96 (validated)
let-7i-5p	TLR4, IL-6, RAS	Mitogen-activated protein kinase (MAPK)	Unchecked chronic hyper-inflammation	94 (validated)

4. Discussion

The molecular findings of this study demonstrate that subclinical trypanosomiasis is not an immunologically passive state, but rather a phase of highly coordinated, post-transcriptional immune remodelling driven by circulating exosomes (Choi *et al.,* 2024). The massive upregulation of exosomal miR-21-5p observed in our cohort represents a critical molecular step in the induction of host T-cell anergy and exhaustion. Bioinformatic analysis confirmed that miR-21-5p directly targets and suppresses Phosphatase and Tensin Homolog (PTEN) and Programmed Cell Death 4 (PDCD4) (Zabeti Touchaei *et al.,* 2024). Under normal physiological conditions, PTEN acts as a vital negative regulator of the Phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling cascade, which ensures controlled cellular activation and survival. When exosomal delivery of miR-21-5p suppresses PTEN, the PI3K/Akt pathway becomes chronically, sub-lethally activated. This continuous, low-level signaling deprives T lymphocytes of normal metabolic rest phases, accelerating a state of cellular senescence and driving the hyper-expression of surface PD-1 coreceptors, exactly as documented in our flow cytometric metrics. This pathway modification is further exacerbated by the loss of PDCD4, which normally stabilizes mRNA translation during acute immune activation; its suppression leaves the T-cell highly vulnerable to apoptotic and anergic stimuli (Wang *et al.,* 2025).

Simultaneously, the marked elevation of exosomal miR-155-5p plays a complex, dual role in this pathological timeline. While miR-155-5p is traditionally recognized as a pro-inflammatory microRNA necessary for early classical macrophage activation and effective Th1 polarization, its sustained, long-term expression during the subclinical phase shifts its functional output (Xu *et al.,* 2022). By targeting Src Homology 2 Domain-Containing Inositol 5-Phosphatase 1 (SHIP1) and Suppressor of Cytokine Signaling 1 (SOCS1), persistent exosomal miR-155-5p permanently disrupts the negative feedback loops that regulate JAK/STAT signaling. This disruption leads to an uncontrolled, chronic pro-inflammatory signaling cascade (Łaba *et al.,* 2025). Over time, this unremitting hyper-inflammatory background causes severe functional exhaustion of effector lymphocytes, driving them into a terminal differentiation state marked by the co-expression of PD-1 and TIM-3 coreceptors. This molecular phenotype is highly indicative of complete functional shutdown.

In stark contrast to these upregulated pathways, the profound suppression of exosomal miR-146a-5p and let-7i-5p highlights a critical failure in host immunoregulatory mechanisms. Exosomal miR-146a-5p normally functions as a major molecular brake on the innate and adaptive immune responses by targeting Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6) and Interleukin-1 Receptor-Associated Kinase 1 (IRAK1) (Liao *et al.,* 2022). Under healthy conditions, this interaction suppresses excess NF-κB activation, preventing systemic immunopathology. The dramatic loss of circulating exosomal miR-146a-5p observed in subclinical hosts completely releases this brake, allowing unchecked TRAF6/IRAK1 translation and continuous NF-κB activation. This loss of regulation removes the host's capacity to induce endotoxin tolerance or metabolic recovery, trapping tissue-resident and circulating immune cells in a state of chronic inflammatory stress that accelerates cellular exhaustion (Robinson *et al.,* 2016).

Similarly, the downregulation of let-7i-5p removes post-transcriptional repression from Toll-Like Receptor 4 (TLR4) and Interleukin-6 (IL-6) transcripts. This loss of control permits the continuous translation of baseline pro-inflammatory cytokines, even when actual systemic parasite counts remain below the limit of microscopic detection. This continuous, low-grade inflammatory state provides the constant, systemic antigenic pressure required to drive helper and cytotoxic T-cell populations into a state of profound exhaustion. These insights indicate that exosomal microRNA signatures can serve as a highly accurate liquid biopsy panel, reflecting the internal state of host immune exhaustion long before macroscopic clinical signs emerge (La Marca *et al.,* 2017).

5. Conclusion

This research demonstrates that circulating exosomal microRNA signatures can serve as highly sensitive, early-stage molecular predictors of immune exhaustion during subclinical trypanosomiasis. High-throughput sequencing and targeted flow cytometric profiling confirmed that a highly specific panel of four core exosomal microRNAs, miR-21-5p, miR-155-5p, miR-146a-5p, and let-7i-5p, undergoes profound dysregulation during the subclinical phase. These liquid biopsy markers are tightly correlated with the upregulation of key cellular checkpoint coreceptors (PD-1, CTLA-4, and TIM-3) and the targeted post-transcriptional modification of core immunological pathways, including the PI3K/Akt/mTOR and NF-κB cascades. By providing a non-invasive look into the host's changing immune status, these exosomal signatures offer a powerful tool to bridge the diagnostic gap in subclinical disease monitoring. This molecular approach enables targeted therapeutic intervention to preserve host immune function and resilience well before unmitigated systemic collapse occurs.

Declarations

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