MicroRNAs AS BIOMARKERS OF LIMB ISCHEMIA

MikroRNA ako biomarkery končatinovej ischémie

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Abstract
Introduction. Limb ischemia represents a continuum from chronic hypoperfusion to acute arterial occlusion, with severe forms associated with high morbidity and mortality. Reliable molecular markers reflecting the severity of ischemic muscle injury are currently lacking, and the role of microRNAs in advanced skeletal muscle ischemia remains insufficiently characterized.
Patients and Methods. This study was based on a secondary analysis of publicly available RNA sequencing data (dataset GSE120642). Gastrocnemius muscle biopsies from healthy controls (n=15), patients with intermittent claudication (n=20), and patients with critical limb ischemia (n=16) were analyzed. Differential gene expression was assessed using DESeq2. Selected microRNAs were evaluated by receiver operating characteristic (ROC) curve analysis and combined into a multivariate logistic regression model. Functional enrichment of experimentally validated microRNA targets from the miRTarBase database (n=418) was performed using the MSigDB Hallmark collection.
Results. Transcriptomic analysis revealed a clear molecular gradient corresponding to ischemic severity. Four microRNAs (miR-3606, miR-198, miR-1244-2, and miR-133b) exhibited consistent upregulation in critical limb ischemia and demonstrated strong discriminatory ability between severe ischemia and both healthy muscle and intermittent claudication (area under the curve = 0.97 – 0.98). Enrichment analysis highlighted pathways related to apoptosis, hypoxia, inflammatory signaling, and tissue remodeling.
Conclusion. A four-microRNA panel associated with advanced ischemic muscle injury was identified, including two microRNAs (miR-3606 and miR-1244-2) not previously linked to this condition. These findings provide new molecular insight and support further investigation of microRNAs as potential prognostic biomarkers in limb ischemia *(Tab. 1, Fig. 4, Ref. 19). Text v PDF www.lekarskyobzor.sk.*

KEY WORDS: limb ischemia, ischemic injury, microRNA, skeletal muscle, biomarkers, gene expression profiling.

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Abstrakt
Úvod. Ischémia končatín predstavuje kontinuum od chronickej hypoperfúzie až po akútnu artériovú oklúziu, pričom jej závažné formy sú spojené s vysokou morbiditou a mortalitou. Spoľahlivé molekulové markery odrážajúce závažnosť ischemického poškodenia svalového tkaniva v súčasnosti chýbajú a úloha mikroRNA v pokročilej ischémii kostrového svalstva zostáva nedostatočne charakterizovaná.
Pacienti a metódy. Táto štúdia bola založená na sekundárnej analýze verejne dostupných údajov z RNA sekvenovania (dataset GSE120642). Analyzované boli biopsie m. gastrocnemius od zdravých kontrol (n = 15), pacientov s intermitentnou klaudikáciou (n = 20) a pacientov s kritickou ischémiou končatín (n = 16). Diferenciálna expresia génov bola hodnotená pomocou nástroja DESeq2. Vybrané mikroRNA boli analyzované pomocou kriviek prevádzkovej charakteristiky prijímača a následne zlúčené do multivariačného logistického regresného modelu. Funkčné obohatenie experimentálne validovaných cieľových génov mikroRNA z databázy miRTarBase (n = 418) bolo vykonané s využitím kolekcie MSigDB Hallmark.
Výsledky. Transkriptomická analýza odhalila jasný molekulový gradient zodpovedajúci závažnosti ischémie. Štyri mikroRNA (miR-3606, miR-198, miR-1244-2 a miR-133b) preukazovali konzistentnú upreguláciu pri kritickej ischémii končatín a preukázali vysokú rozlišovaciu schopnosť medzi závažnou ischémiou, zdravým svalovým tkanivom a intermitentnou klaudikáciou (plocha pod krivkou = 0,97 – 0,98). Analýza obohatenia identifikovala dráhy súvisiace s apoptózou, hypoxiou, zápalovou signalizáciou a remodeláciou tkaniva.
Záver. Bol identifikovaný panel štyroch mikroRNA spojený s pokročilým ischemickým poškodením svalového tkaniva, pričom dve mikroRNA (miR-3606 a miR-1244-2) neboli s týmto stavom doteraz spájané. Zistenia poskytujú nový molekulárny pohľad na patofyziológiu ischémie končatín a podporujú ďalší výskum mikroRNA ako potenciálnych prognostických biomarkerov pri ischémii končatín *(tab. 1, obr. 4, lit. 19). Text v PDF www.lekarskyobzor.sk.*

Kľúčové slová: končatinová ischémia, ischemické poškodenie, mikroRNA, kostrový sval, biomarkery, profilovanie génovej expresie.
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Introduction

Limb ischemia represents a spectrum of vascular disorders ranging from chronic perfusion impairment to acute arterial occlusion, with progressive consequences for skeletal muscle viability (1). While acute limb ischemia (ALI) is associated with a high risk of limb loss and systemic complications, chronic and critical limb ischemia (CLI) reflect prolonged and cumulative ischemic injury characterized by hypoxia, inflammation, and structural tissue damage (2). Despite advances in surgical and endovascular treatment, clinical outcomes across all forms of limb ischemia remain highly variable and de-

pend largely on the extent of underlying tissue injury and the biological response to ischemia and reperfusion (3). Current diagnostic approaches primarily rely on clinical assessment and imaging techniques, providing limited insight into the molecular severity of muscle damage (1). As a result, reliable molecular biomarkers capable of reflecting ischemic burden, inflammatory activation, and tissue viability are needed to improve disease characterization, risk stratification, and prognostic evaluation across the spectrum of limb ischemia (2, 4). MicroRNAs (miRNAs) represent promising candidates for such biomarkers due to their stability in blood circulation and their regulatory role in hypoxia, inflammation, and stress-related pathways (5). However, miRNA profiles associated with severe skeletal muscle ischemia remain insufficiently characterized. Therefore, using CLI as a biologically relevant model of advanced ischemic injury, this study aimed to identify miRNAs associated with severe muscle damage and evaluate their relevance across different stages of limb ischemia.

Patient cohort and methodology

This study represents a secondary analysis of publicly available transcriptomic data obtained from the Gene Expression Omnibus (GEO) database under accession number GSE120642. The dataset includes RNA sequencing profiles of gastrocnemius muscle biopsies collected from three clinical groups: healthy older adults without peripheral artery disease (n=15), patients with intermittent claudication (IC; n=20), and patients with critical limb ischemia (CLI; n=16) undergoing major limb amputation. The analyzed samples represent progressive stages of lower limb ischemia. Demographic and clinical characteristics of the study participants, including age and sex distribution, are described in detail in the original publication (6).

Normalized transcript count matrices provided by the original study were used for all downstream analyses. Data quality was evaluated by unsupervised dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP) to assess global sample structure. Differential gene expression analysis was performed using the DESeq2 package in R (version 4.2.2). Both UMAP and DESeq2 were implemented via the GEO2R interface. Three predefined pairwise comparisons were analyzed: CLI vs healthy controls, CLI vs IC, and IC vs healthy controls. Genes with an adjusted p value <0.05 were considered significantly differentially expressed. Correction of p values was done using the Benjamini-Hochberg correction.

Candidate miRNAs were selected from the differential expression results based on predefined selection criteria. Individual miRNAs were evaluated using receiver operating characteristic (ROC) curve analysis. Selected miRNAs were subsequently combined into a multivariate logistic regression model. Expression values were standardized before model fitting. Model optimization was performed using grid search and stratified five-fold cross-validation. Optimal classification thresholds were determined using the Youden index, which represents the optimal cut-off value that maximizes the sum of sensitivity and specificity on the ROC curve. Model performance was assessed using ROC curves and confusion matrix analysis. Statistical analyses were conducted in Python (version 3.12) using standard scientific libraries, including pandas, NumPy, scikit-learn, matplotlib, and gseapy. Experimentally validated target genes of selected miRNAs were retrieved from miRTarBase. Over-representation analysis was performed using Fisher's exact test with the Molecular Signature Database (MSigDB) Hallmark gene set collection implemented via the gseapy library. Pathways were filtered based on adjusted p values, as determined by the Benjamini-Hochberg correction, and gene set size (limit = 500 genes) to reduce redundancy and dilution of biological signal.

This study was based exclusively on anonymized, publicly available data. No additional ethical approval was required for the present secondary analysis.

Results

Differential expression analysis of gastrocnemius muscle samples from HC, patients with IC, and CLI identified widespread transcriptomic changes across the ischemic spectrum. Using DESeq2 (adjusted p value <0.05), 16 261 transcripts were differentially expressed in at least one comparison.

The principal finding was a set of 1 373 transcripts consistently dysregulated in both CLI vs healthy controls and IC vs CLI contrasts, indicating progressive and concordant regulation with increasing ischemic severity (Fig. 1). In contrast, only 16 transcripts differed only between healthy controls and IC, suggesting relative transcriptomic stability in early disease stages. Unsupervised UMAP analysis confirmed clear separation of clinical groups, with IC samples occupying an intermediate position between healthy controls and CLI. These results identify the 1 373 shared transcripts as a core molecular signature of progressive ischemic muscle injury and provide the basis for subsequent miRNA-focused analyses.

Based on differential expression profiles in CLI muscle, eight miRNAs were identified that showed a consistent regulation pattern across both CLI contrasts. Four miRNAs (miR-12136, miR-1307, miR-5006 and miR-133a-2) were significantly downregulated in CLI, whereas another four (miR-3606, miR-198, miR-1244-2 and miR-133b) were strongly upregulated (Tab. 1). The direction and magnitude of $\log_2$ fold-changes were highly concordant between CLI vs IC and CLI vs healthy controls, indicating that these miRNAs represent stable markers of advanced ischemic injury rather than group-specific artefacts.

Figure 1. Results of differential expression analysis using the DESeq2 and UMAP tools in the GEO2R interface. Padj – adjusted p-value, UMAP – Uniform Manifold Approximation and Projection, nbrs – number of neighboring samples used for clustering, HC – healthy controls, IC – intermittent claudication, CLI – critical limb ischemia.

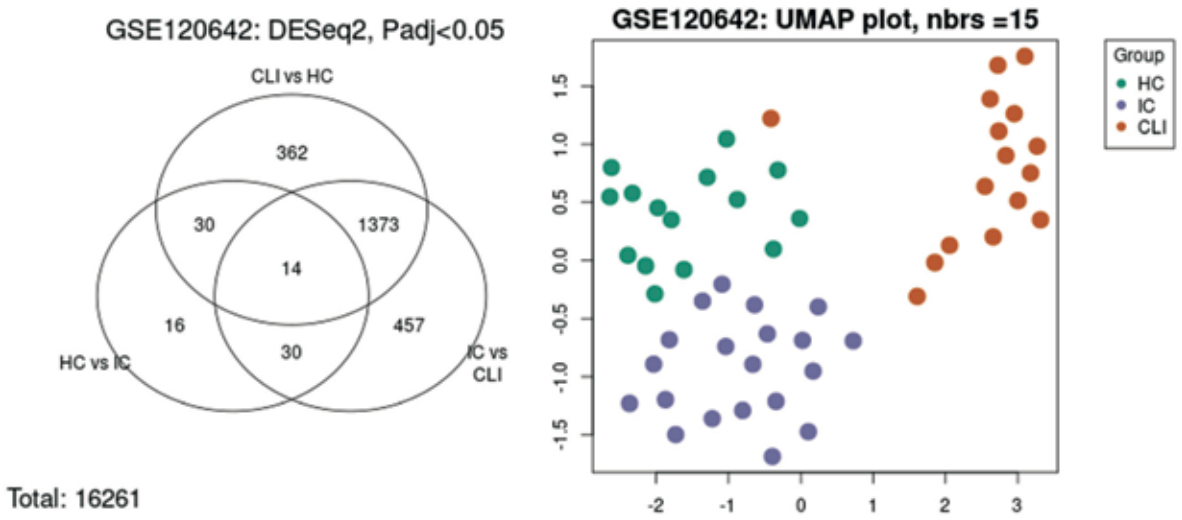


Table 1. Panel of microRNAs with consistent direction of dysregulation.

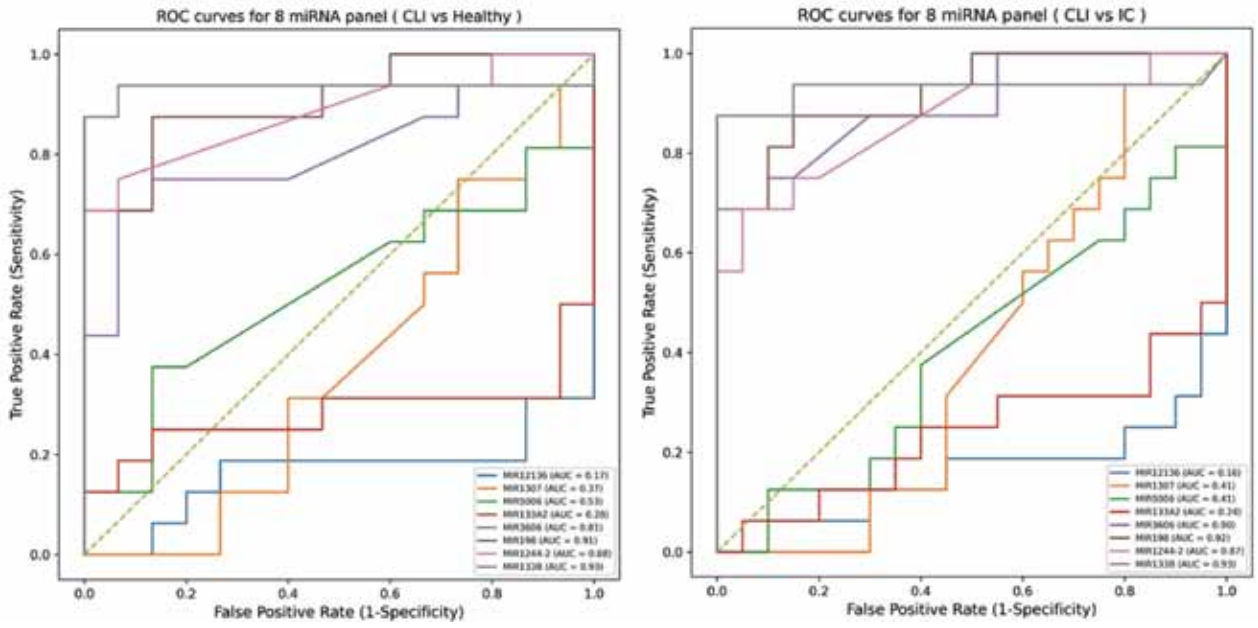
Gene	Symbol	log_2FC (CLI vs IC)	log_2FC (CLI vs Healthy)	Expression trend in CLI
MIR12136	miR-12136	−1.37	−1.42	Downregulated
MIR1307	miR-1307	−1.74	−2.08	Downregulated
MIR5006	miR-5006	−1.16	−1.02	Downregulated
MIR133A2	miR-133a-2	−1.58	−1.46	Downregulated
MIR3606	miR-3606	1.94	1.67	Upregulated
MIR198	miR-198	1.5	1.22	Upregulated
MIR1244-2	miR-1244-2	1.57	1.64	Upregulated
MIR133B	miR-133b	2.84	2.3	Upregulated

CLI – critical limb ischemia, IC – intermittent claudication

To evaluate the discriminative performance of the initial 8-miRNA panel, ROC curves were generated for each miRNA in the CLI vs healthy controls and CLI vs IC contrasts (Fig. 2). Among them, miR-198, miR-3606, miR-1244-2, and miR-133b showed the strongest and most consistent discrimination, with area under the curve (AUC) values up to 0.93 for CLI vs healthy controls and 0.86 for CLI vs IC. The remaining miRNAs exhibited poor individual performance (AUC ≤ 0.53). These findings supported the selection of a core 4-miRNA signature for subsequent multivariate modeling.

To identify a clinically relevant miRNA signature associated with severe ischemic muscle injury, the four

Figure 2. Receiver operating characteristic (ROC) curves for a panel of 8 microRNAs (miRNAs) **evaluated for diagnostic performance in distinguishing patients with critical limb ischemia** (CLI) **from intermittent claudication** (IC) **and healthy controls.** AUC – Area under the Curve.



most informative miRNAs (miR-3606, miR-198, miR-1244-2 and miR-133b) were combined into a single diagnostic panel. This 4-miRNA signature showed excellent ability to distinguish patients with CLI from both healthy controls and patients with IC (Fig. 3).

The model achieved very high diagnostic accuracy, with an area under the ROC curve of 0.98 for CLI versus healthy controls and 0.97 for CLI versus IC. Using optimal decision thresholds, sensitivity reached 94 %, while specificity was 100 % in both comparisons. Importantly, no false-positive classifications were observed, and only one CLI case was misclassified in each comparison, indicating reliable identification of severe ischemic tissue damage.

The corresponding confusion matrices (Fig. 4) further illustrate this behavior. In both contrasts, the model generated no false-positive predictions, correctly identifying all non-CLI samples. False-negative classifications were rare. Overall, these findings demonstrate that the selected 4-miRNA signature consistently reflects the severity of muscle ischemia and can clearly differentiate advanced, non-viable ischemic tissue from both healthy muscle and chronically ischemic but viable muscle.

To explore the biological relevance of the 4-miRNA signature, over-representation analysis of experimentally validated target genes (n=418) was performed using the MSigDB Hallmark collection. The analysis identified

Figure 3. Receiver operating characteristic (ROC) **curves showing the diagnostic performance of an optimized logistic regression model based on a 4-miRNA core signature for discrimination of patients with critical limb ischemia** (CLI) **from intermittent claudication** (IC) **and healthy controls.** AUC – Area under the Curve.

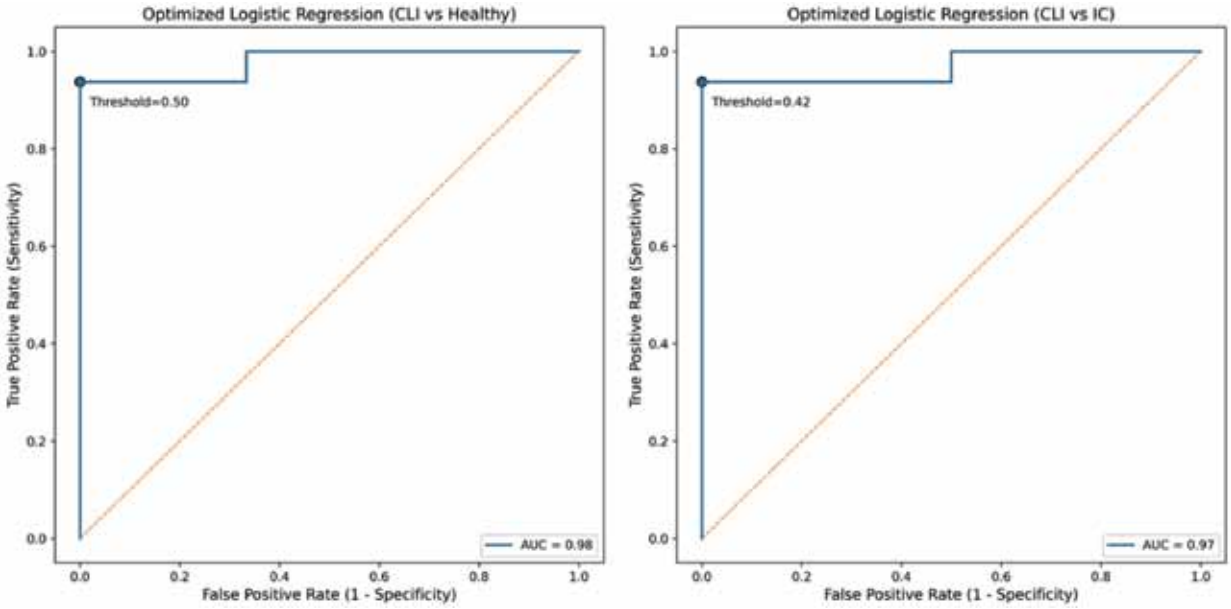
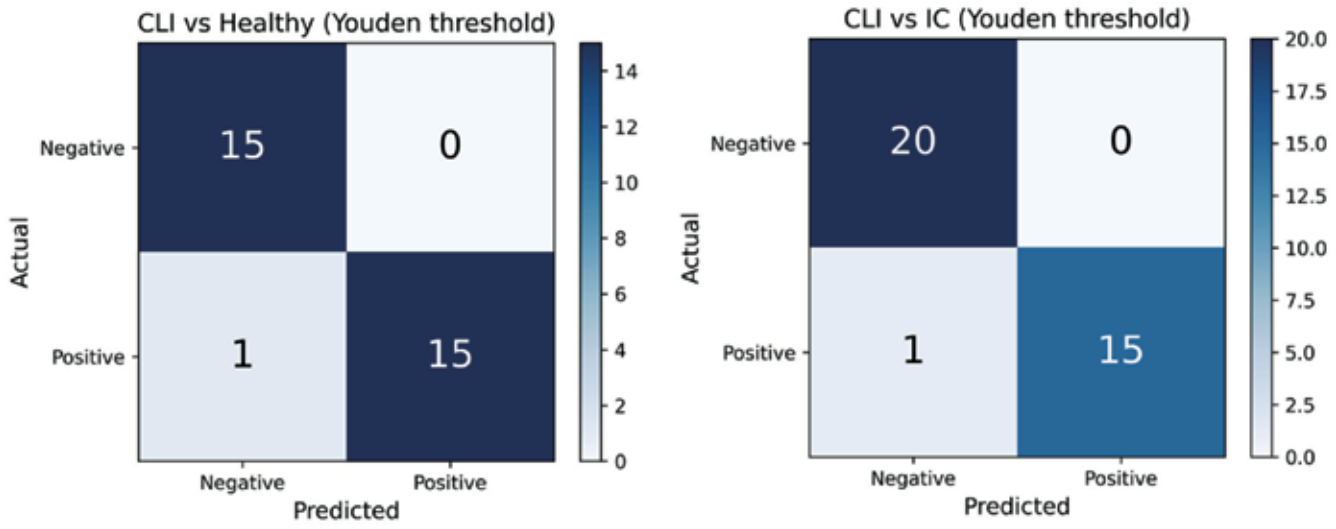


Figure 4. Confusion matrices illustrating the classification performance of the optimized logistic regression model based on the 4-miRNA core signature for discrimination of patients with critical limb ischemia (CLI) **from healthy individuals and intermittent claudication** (IC)**, using the Youden index–derived optimal threshold.**



a compact and biologically coherent set of pathways associated with advanced ischemic muscle injury.

The most significantly enriched pathway was apoptosis (adjusted p = 6.83×10^{-5}), indicating extensive activation of programmed cell death in severely ischemic tissue. Significant enrichment was also observed for the phosphoinositide 3-kinase–protein kinase B–mechanistic target of rapamycin signaling pathway (PI3K–AKT–mTOR) (adjusted p = 0.0080), suggesting engagement of stress-related survival and metabolic adaptation mechanisms. Additional pathways included transforming growth factor β (TGF-β) signaling (adjusted p = 0.0133) and androgen response (adjusted p = 0.0133). Several pathways showed moderate but consistent enrichment at comparable significance levels, including hedgehog signaling, angiogenesis, hypoxia, apical junction organization, epithelial–mesenchymal transition, and the p53 pathway (all adjusted p = 0.0432).

Discussion

Limb ischemia is associated with significant morbidity and adverse clinical outcomes, which are strongly influenced by the extent of ischemic muscle injury and the subsequent biological response to hypoxia and reperfusion (1, 4, 7). Although clinical assessment and imaging are fundamental for disease evaluation, they provide limited insight into molecular processes underlying tissue damage, inflammation, and repair (4, 8) Therefore, the identification of molecular markers reflecting ischemic burden remains an important unmet need across different forms of limb ischemia.

In this study, critical limb ischemia was used as a biologically relevant model of advanced ischemic muscle injury, enabling the investigation of miRNA-associated molecular changes across different stages of limb ischemia. Transcriptomic analysis revealed a clear molecular gradient from healthy muscle through IC to CLI, consistent with previously described patterns of progressive hypoxia, inflammation, and structural remodeling in chronically ischemic skeletal muscle. These observations were extended by demonstrating that specific miRNAs exhibit monotonic regulation across this ischemic continuum. Among the identified miRNAs, miR-3606, miR-198, miR-1244-2, and miR-133b were found to be most consistently associated with ischemic severity. Members of the miR-133 family have previously been implicated in skeletal muscle injury and regeneration, supporting their biological relevance in ischemic conditions (9, 10). miR-198 has been associated with inflammatory signaling and stress responses in vascular and epithelial tissues (11, 12). In contrast, miR-3606 (13) and miR-1244-2 (14) have been reported only in biologically related contexts, such as wound healing or hypercholesterolemia. The association of these miRNAs with severe ischemic muscle injury is considered a novel finding of the present study. The combined analysis of these four miRNAs demonstrated robust discrimination between CLI and both healthy muscle and IC samples. Notably, the ability to distinguish CLI from IC indicates sensitivity to differences between non-viable and viable ischemic tissue, a distinction of clinical relevance in acute ischemic settings. Rather than proposing a diagnostic tool for CLI itself, which is clinically apparent, the results support the interpretation that the identified miRNAs reflect fundamental biological processes related to ischemic tissue damage. Functional enrichment analysis of experimentally validated miRNA targets further supported the biological plausibility of the identified signature. Enrichment of pathways related to apoptosis, hypoxia, PI3K–AKT–mTOR signaling, TGF-β signaling, hedgehog signaling, and p53-mediated stress responses agree with mechanisms of ischemia–reperfusion injury described in experimental and clinical studies (15, 16). These pathways are centrally involved in myocyte death, inflammatory activation, cellular stress adaptation, and tissue remodeling, reinforcing the relevance of the miRNA signature to ischemic pathophysiology (17–19).

This study has several limitations. The analysis was based on cross-sectional transcriptomic data obtained from chronically ischemic muscle tissue, which may not fully reflect dynamic molecular changes occurring during different phases of limb ischemia. However, critical limb ischemia provides a relevant model of advanced ischemic injury. In addition, the study focused on tissue-derived miRNAs, while circulating miRNA levels were not evaluated.

Conclusion

This study identifies a four-miRNA signature associated with advanced ischemic skeletal muscle injury, derived from transcriptomic profiling of CLI. The proposed signature includes two microRNAs (miR-3606 and miR-1244-2) that have not previously been implicated in this condition. A key finding is the consistent and proportional regulation of these miRNAs across the ischemic severity spectrum, enabling discrimination between viable and severely damaged muscle tissue and reflecting the underlying biological extent of ischemic injury. From a clinical perspective, the observed miRNA expression patterns appear to mirror fundamental pathological processes of limb ischemia, including apoptosis, hypoxic stress, inflammatory activation, and tissue remodeling. Although the present analysis is based on tissue-derived transcriptomic data, the identified miRNAs constitute biologically plausible candidates for future blood-based biomarkers, with potential utility in risk stratification and prognostic assessment of patients following revascularization procedures. Collectively, these findings provide a focused molecular framework for further translational studies aimed at validating miRNA-based markers in clinically relevant ischemic settings.*

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Informed consent: Informed consent was not required for the study.

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