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LncRNAs upregulated in insulin resistance are downregulated by metformin in a liver cell line

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Abstract

Insulin resistance (IR) is a key contributor to the development of metabolic diseases, and metformin has been shown to help mitigate IR. Long non-coding RNAs (lncRNAs) are emerging as important regulators in metabolic disorders. This study aimed to investigate the differential expression of lncRNAs in IR and assess the impact of metformin on these lncRNAs. Using the Huh7 cell line to model IR (Huh7-IR), we treated the cells with metformin (Huh7-IR+Metf). Microarray analysis, followed by bioinformatic analysis in RStudio, identified 127 downregulated and 109 upregulated lncRNAs, among which 60 showed reduced expression following metformin treatment in Huh7-IR cells. Notably, the upregulated lncRNAs HOX transcript antisense RNA (HOTAIR), long intergenic non-protein coding RNA, muscle differentiation 1 (LINCMD1) and Prader-Willi region non-protein coding RNA 2 (PWRN2) were found to be associated with genes involved in the insulin signaling pathway. These three lncRNAs were further validated using real-time RT-PCR. This study highlights the differential expression of lncRNAs in IR and their modulation by metformin. Specifically, metformin restores the expression of lncRNAs that were deregulated in IR, including HOTAIR, LINCMD1, and PWRN2, likely through the regulation of critical biological processes and signaling pathways associated with IR. In conclusion, our findings demonstrate that metformin modulates the expression of key lncRNAs, including HOTAIR, LINCMD1, and PWRN2, which are deregulated in insulin resistance. This regulation likely occurs through the modulation of critical signaling pathways, such as NFκB and AMPK, suggesting that targeting lncRNAs could offer new therapeutic avenues for managing IR and related metabolic disorders.

Keywords: Insulin resistance, Metformin, HOTAIR, LINCMD1, lncRNAs, PWRN2.

1. Introduction

Insulin resistance (IR) arises when cells exhibit a diminished response to insulin. RI contributes to the development of metabolic syndrome, type-2 diabetes (T2D), non-alcoholic steatohepatitis, and cardiovascular diseases. Factors associated with IR development include obesity, hypertension, and inflammation [1]. Inflammation is linked to elevated levels of proinflammatory cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNFα), which are upregulated by nuclear factor kappa B (NFκB) [2]. Additionally, mutations, post-translational modifications of the insulin receptor (INSR), and the deregulation of downstream effector molecules of the INSR have been associated with IR [1, 2]. Epigenetic factors, including long non-coding RNAs (lncRNAs), have also been implicated in the IR [3]. lncRNAs are known to regulate gene expression at both the transcriptional and post-transcriptional levels [4].

Several studies have identified a relationship between lncRNAs and IR as well as other metabolic alterations associated with insulin. The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been found to positively regulate sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor that controls the expression of hepatic lipogenic genes. Increased expression and activity of SREBP-1c contribute to hepatic lipid accumulation and IR. MALAT1 also activates the c-Jun N-terminal kinase (JNK) pathway, leading to the inhibition of insulin receptor substrate-1 (IRS-1) and protein kinase B (AKT) phosphorylation [5, 6]. LncRNA MEG3 promotes IR by significantly increasing the expression of forkhead box protein O1 (FOXO1), which in turn enhances the transcription of the gluconeogenic enzymes glucose 6-phosphatase and phosphoenolpyruvate car-

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boxykinase-1. Additionally, MEG3 functions as an endogenous RNA of miR-181, a miRNA that suppresses early growth response 2 (EGR2) expression, which is elevated in palmitate-induced IR [7, 8]. Similarly, the upregulated HOX transcript antisense RNA (HOTAIR) is suggested to promote hepatic IR by inhibiting sirtuin 1 (SIRT1) expression and the AKT/GSK3 pathway [9]. High levels of HOTAIR in patients with type 2 diabetes (T2D) have been shown to help discriminate uncontrolled comorbidities related to the disease, suggesting its potential use as a biomarker for prognosis or early diagnosis [10]. Other lncRNAs implicated in IR include GASH, MIAT (Gomafu), Risa, and H19 [3]. However, the mechanisms by which lncRNAs participate in IR remain unclear.

Metformin is commonly used to enhance insulin sensitivity, alongside a healthy diet, weight management, and physical activity. Its hypoglycemic effect primarily stems from inhibiting hepatic gluconeogenesis through complex I inhibition in the mitochondrial respiratory chain. This leads to an increased AMP/ATP ratio, activating the AMP-activated protein kinase (AMPK) pathway. Additionally, metformin inhibits complex IV, resulting in decreased glycerol-3-phosphate dehydrogenase activity and an altered cytosolic redox state (NADH: NAD+), ultimately reducing gluconeogenesis from lactate [11]. In this study, the differential expression of lncRNAs in IR and after metformin treatment was evaluated, and the expression of three lncRNAs upregulated in IR and downregulated with metformin were validated.

2. Materials and methods

2.1. Cell culture, induction of insulin resistance and metformin treatment

Huh7 cells, derived from hepatocellular carcinoma, are well-known for their metabolic similarity to primary human hepatocytes. This is a permanent and commercially available cell line that was established in 1982. The Huh7 cell line used in this study was generously provided by Dr. María Concepción Gutiérrez Ruíz from Universidad Autónoma Metropolitana, CDMX, Mexico. Huh7 cells were cultured at 37 ºC and 5% CO_2 in William's E (WE) medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS) and antibiotic-antimycotic at 1% (containing 100 U/mL penicillin G sodium, 1000 µg/mL de streptomycin and 0.25 µg/mL amphotericin B). The cells were seeded at a density of $2x10^5$ cells/mL and exposed to a glucose concentration and human insulin (Sigma-Aldrich, St. Louis, MO, USA) to induce IR (Huh7-IR). Additionally, a group of Huh7-IR cells was treated with 2 mM metformin (Sigma-Aldrich, St. Louis, MO, USA) (Huh7-IR+Metf) for 24 h. The verification of the induction of IR and the effect of metformin was analyzed by the assays of glucose uptake, intracellular glycogen and lipid content, glucose production, and the analysis of the phosphorylation of tyrosine 1885 in the β subfraction of the insulin receptor, pAMPK, and GLUT-2 levels, methodology that has been previously described elsewhere by our working group [12]. In this study, we used three groups of cells: 1) control Huh7 (Huh7-Ctrl), 2) Huh7 cells with insulin resistance (Huh7-IR), and 3) Huh7 cells with IR treated with metformin (Huh7-IR+Metf).

2.2. Total RNA extraction

The cells were placed on ice, washed twice with cold phosphate-buffered saline (PBS), and then centrifuged. Next, we harvested the cells in 1.5 mL tubes containing 500 µL of TRizol-LS reagent (Thermo Fisher Scientific, Waltham, MA, USA) for RNA extraction, following the manufacturer's instructions.

2.3. Microarray hybridization

Isolated total RNA underwent a series of steps for expression profiling using the Clariom D Human Assay (ThermoFisher, Scientific, Waltham, MA, USA). First, each purified RNA sample was transcribed into double-strand cDNA, followed by cRNA synthesis and biotin-labeling. The resulting cRNA was then purified using an Affymetrix magnetic bead protocol. Next, the samples were hybridized to Clariom D arrays, which detect over 540,000 transcripts. After a 16-hour incubation, the arrays were scanned using the GeneChip Scanner 3000. Primary data analysis was performed using Affymetrix Expression Console and Transcriptome Analysis Console software.

2.4. Differential gene expression analysis

The microarray data were processed using RStudio v4.2.1, along with several Bioconductor packages, including affycoretools, oligo, and pd.clariom.d.human (https://bioconductor.org/packages/release/data/annotation/html/pd.clariom.d.human.html). Quality control and data normalization were performed based on the Bioconductor package recommendations. To identify differentially expressed lncRNAs and mRNAs, we compared Huh7-IR vs. Huh7-Ctrl and Huh7-IR+Metf vs. Huh7-IR using the 'limma' software this experimental design allows assessment: 1) changes in expression profile linked to insulin resistance, and 2) the metformin-induced changes in expression profile on insulin-resistant cells. For each gene, we applied a student's t-test and calculated the logarithm of differential expressions. The Benjamini and Hochberg method was then used to adjust p-values and reduce false positives. Differentially expressed mRNAs in Huh7-IR cells were identified using a log2 fold-change (logFC) $\geq$ 0.5 and $p \leq 0.05$. Differentially expressed lncRNAs were selected with a logFC $\geq$ 1.45, $p \leq 0.05$, and adjusted $p \leq$ 0.05.

2.5. Construction of protein-protein and lncRNAs-mRNAs interaction networks

The protein-protein interaction (PPI) network was constructed using the STRING tool within Cytoscape software (version 3.9.1.). Additionally, we employed CytoHubba algorithms, which complement Cytoscape by utilizing Maximal Clique Centrality (MCC) to identify central gene nodes.

2.6. Functional enrichment and protein-protein interaction networks

We conducted functional enrichment analysis and explored protein-protein interaction networks using the STRING v.11.5 database (https://string-db.org). For pathway enrichment analysis, we integrated mRNAs that were differentially expressed in Huh7-IR cells and genes associated with previously described lncRNAs from publications. To perform this analysis, we utilized several tools: ShinyGO 0.76.3 (http://bioinformatics.sdstate.edu/go/), LNCipedia version 5.2 (https://lncipedia.org/), GENCODE (https://www.gencodegenes.org/), GeneCards

(https://www.genecards.org/), and Ensembl (https://www.ensembl.org/index.html). The selection of enriched pathways was based on the database-integrated false discovery rate (FDR) with a significance threshold of $p \leq 0.05$.

2.7. Validation of candidate lncRNAs by real-time RT-qPCR

Total RNA was extracted from Huh7 cells from all three conditions, using 500 µL of TRizol-LS reagent. The cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Briefly, the 2X RT Master Mix was prepared for each reaction by combining 2 µL of 10X RT buffer, 0.8 µL of 25X dNTP mix (100 mM), 2 µL of 10X RT random primers, 1 µL of reverse transcriptase (50 U/µL), and 4.2 µL of RNase-free water. Subsequently, 500 ng of total RNA, diluted in 10 µL of RNase-free water, was added to the reaction and cDNA amplification was performed using a thermal cycler (Axygen Scientific, Union City, CA, USA). The expression of lncRNAs was determined using gene expression assays with the following TaqMan probes (Thermo Fisher Scientific, Waltham, MA, USA): *HOTAIR* (Hs05502358_s1), LINCMD1 (Hs00416173_m1), and PWRN2 (4351372 Hs05027496_gH). The final volume of the reaction mixture was 10 µL containing 5 µL of FastAdvanced Master mix, 0.5 µL of TaqMan probes, 3.5 µL of nuclease-free water, and 1 µL of cDNA. The reaction was performed on the QuantStudio 5 real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The PCRs were performed in triplicate, using *GAPDH* gene (4331182 Hs02786624_g1) as control. Finally, the relative expression calculations of the lncRNAs performed by the method $2^{-\Delta\Delta Ct}$.

2.8. Statistical analysis

One-way ANOVA was used for comparison of means, followed by the Bonferroni test. Graphs were generated using GraphPad Prism v.8.0 software. R v.4.2.1 software was used together with the Bioconductor's limma package to analyze differential expressions. This involved applying linear models and the empirical Bayes model to assess lncRNAs and mRNAs. In addition, the correlation between differentially expressed lncRNAs and mRNAs was explored using Pearson's correlation coefficient (PCC). Interaction networks of positively and negatively correlated genes ($PCC > 0.8$; $p \leq 0.05$) were constructed using Cytoscape v.3.9.1 software.

3. Results

3.1. Differential expression analysis of lncRNAs and mRNAs

Through microarray analysis, 109 upregulated and 127 downregulated lncRNAs were identified in Huh7-IR cells compared with Huh7-Ctrl. Interestingly, upon metformin treatment, the 109 lncRNAs that were upregulated in IR cells were found to be downregulated. Furthermore, among the lncRNAs that were downregulated in IR cells, only 60 of them were upregulated with metformin. Notably, the lncRNAs HOTAIR, LINCMD1, and PWRN2 were found to be upregulated in IR cells and were related to nearby genes involved in the insulin signaling pathway, which were selected for further analysis. As mentioned earlier, the expression of these three lncRNAs decreased when IR cells were treated with metformin (Table 1). Additionally, the volcano plots illustrate the changes in the expression of these three lncRNAs under conditions of IR and following treatment with metformin (Figure 1). In our bioinformatics analysis, we also identified 313 mRNAs that were upregulated and 206 that were downregulated in Huh7-IR cells. Interestingly, with metformin treatment, 21 of these mRNAs were downregulated.

Experimental evidence has shown that HOTAIR inhibits the expression of sirtuin-1 (SIRT1), a nicotinamide adenine dinucleotide (NAD+)–dependent protein deacetylase. In differential gene expression analysis, we observed that SIRT1 expression was decreased in Huh7-IR (logFC = -2, $p = 0.006$), while HOTAIR was upregulated. Interestingly, in Huh7-IR+Metf cells, SIRT1 expression increased and HOTAIR decreased (Table 1).

Interestingly, *LINCMD1* is located near the loci encoding interleukins 17A and 17F (*IL17A* and *IL17F*). This proximity suggests that LINCMD1 may interact with these cytokines and potentially participate in the positive regulation of NFκB. To investigate this, we analyzed the expressions of IL17A and IL17F in microarray data. Notably, in Huh7-IR cells, IL17A exhibited a logFC of 0.7 ($p = 0.03$), while in Huh7-IR+Metf cells, its expression significantly decreased (logFC = -1.1, $p = 0.02$). This implies that LINCMD1 might be co-expressed with IL17A,

Table 1. lncRNAs and mRNAs in cells with insulin resistance and its regulation with metformin.

lncRNAs	Huh7-IR*			Huh7-IR+Metf‡		
	logFC	p-value	Padj	logFC	p-value	p-adj
LINCMD1	2.2	< 0.001	0.04	-2.1	0.002	0.01
PWRN2	1.6	0.002	0.05	-0.9	< 0.001	0.005
HOTAIR	1.45	0.001	0.05	-0.7	< 0.001	0.005
mRNAs						
Iinterleukin-17A	0.7	0.03		-1.1	0.02	
Interleukin-17F	0.02	0.9		0.2	0.5	
NFKBIA	-0.9	0.03		1.2	0.01	
Sirtuin-1	-2.09	0.006		2.2	0.02	

Abbreviations: logFC, log-fold change; Padj, adjusted P-value; LINCMD1, long intergenic non-protein coding RNA, muscle differentiation 1; PWRN2, Prader-Willi region non-protein coding RNA 2; HOTAIR, HOX transcript antisense RNA; NFKBIA, Nuclear factor kappa B inhibitor alpha. The p-values were obtained with the Bayes statistical method. * Changes estimates respect to control Huh7 cells. ‡ Changes estimates respect to insulin resistant Huh7 cells.

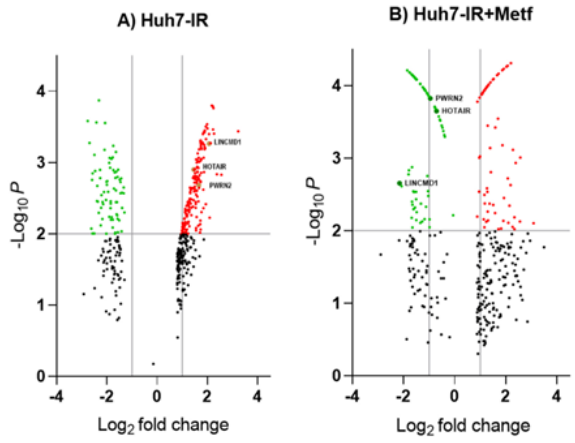

Fig. 1. Change in expression of the lncRNAs in Huh7-IR and Huh7-IR+Metf cells. A) The lncRNAs that were upregulated (red) and the lncRNAs that were downregulated (green) in Huh7-IR are shown ($p \leq 0.05$). B) The lncRNAs that were upregulated (red) and the lncRNAs that were downregulated in Huh7-IR+Metf are shown ($p \leq 0.05$).

potentially contributing to the inflammatory process associated with IR. However, no similar relationship was observed between LINCMD1 and IL17F, as IL17F exhibited low expression levels in both Huh7-IR and Huh7-IR+Metf cells.

PWRN2 has been reported to interact with NFκB inhibitor alpha (NFKBIA), promoting upregulation of NFκB and increased proinflammatory cytokines. Our results show a reduction in the expression of NFKBIA in Huh7-IR cells, whereas in Huh7-IR+Metf cells, NFKBIA expression was elevated. These findings suggest that PWRN2 may influence the transcriptional regulation of NFKBIA and that metformin promotes its transcription (Table 1).

In Huh7-IR cells, we detected a reduction in the expression of genes encoding key proteins in the insulin signaling pathway, including pyruvate dehydrogenase kinase 1 and 2 (PDK1 and PDK2), AKT serine/threonine kinase 2 (AKT2), and phosphatidylinositol-3-kinase (PI3K). However, in Huh7-IR+Metf the expression of these genes, along with SIRT1, was restored. Furthermore, a decrease in the expression of various subunits of AMP-activated protein kinase was observed in Huh7-IR, but metformin treatment led to an increase in their expression (Table 2).

3.2. Construction of protein-protein and lncRNAs-mRNAs interaction networks

In the protein-protein interaction (PPI) network, 70 nodes were analyzed to identify genes with significant interactions. As a result, we identified 10 core genes within the network: IL6, TNF, IL1β, IL17A, interferon regulatory factor 7 (IRF7), TNF, superfamily member 13B (TNFSF13B), TLR7, IL3 receptor subunit alpha (IL3RA), NFKB subunit 2 (NFKB2), and NFKB subunit 1 (NFKB1) (Figure 2). These 10 genes may play a relevant role in IR, given that NFκB (NFKB) and various cytokines have been

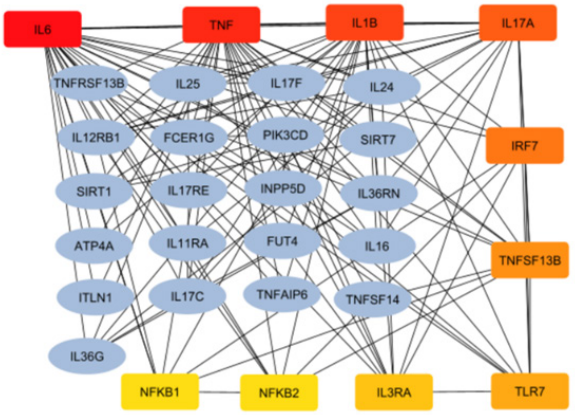

Fig. 2. Protein-protein interaction network of genes with differential expression in Huh7-IR cells. Nodes represent genes and edges represent the connections between them. Rectangles highlight the ten core genes in the network, identified using the MCC (Maximal Clique Centrality) algorithm in CytoHubba, a Cytoscape plugin. The color scale used reflects the MCC scores of the genes, with nodes in red indicating high centrality and nodes in yellow indicating low centrality.

Table 2. Expression of the genes that code for proteins of the insulin signaling pathway and of the genes that code for the AMPK subunits.

Gen	Huh7-IR*		Huh7-IR+Metf*	
	logFC	p-value	logFC	p-value
PI3K	-2.09	< 0.001	1.7	0.01
PDK1	-2.09	0.001	3.1	0.02
PDK2	-3	< 0.001	2.5	< 0.001
AKT2	-1.8	< 0.001	1.7	0.05
STK11	-0.7	0.05	0.6	0.007
PRKAA1	-1.9	< 0.001	2.3	0.05
PRKAA2	-2.5	0.01	2.5	< 0.001
PRKAB1	-1.6	< 0.001	1.6	0.09
PRKAB2	-2.6	< 0.001	2.6	0.009
PRKAG1	-1.5	0.003	1.6	0.03
PRKAG2	-1.4	0.09	1.3	< 0.001

Abbreviations: logFC, log-fold change; *PDK*, pyruvate dehydrogenase kinase; *AKT2*, *AKT* serine/threonine kinase 2; *PI3K*, phosphatidylinosito-l-3-kinase; STK11, serine/threonine kinase 11; *PRKAA*, protein kinase AMP-activated catalytic subunit alpha; *PRKAB, PRK*A subunit beta; *PRKAG, PRKA* subunit gamma. The p-values were obtained with the Bayes statistical method. * Changes estimates respect to control Huh7 cells. ⁑ Changes estimates respect to insulin resistant Huh7 cells.

associated with IR development. Additionally, these genes could be regulated by long non-coding RNAs (lncRNAs). Subsequently, we analyzed the correlation between lncRNAs and mRNAs with differential expression in IR. Notably, HOTAIR, PWRN2, and LINCMD1 were positively correlated with genes encoding interleukins, while showing a negative correlation with SIRT1, AKT2, and genes encoding subunits of the AMPK protein (Figure 3).

3.3. Functional enrichment analysis

Functional enrichment analysis was performed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases for the genes correlated with the selected lncRNAs. The results showed that the up-regulated genes are involved in different pathways, including cytokine-cytokine receptor interaction, Janus-kinase/signal transducer and activator of transcription (JAK-STAT), IR, phosphatidylinositol signaling system, IL-17 signaling, and the toll-like receptor (TLR). Downregulated genes were found in different insulin signaling pathways, such as AMPK, FOXO, insulin, glucagon and PI3K-AKT (Figure 4A). Furthermore, we found that the upregulated genes were associated with several biological processes implicated in the inflammatory response, including cytokine-mediated signaling pathways, cellular responses to cytokine stimuli, cytokine responses, and cytokine production. Conversely, the downregulated genes were linked to lipid and carbohydrate metabolism, protein phosphorylation, and insulin receptor signaling (Figure 4B). Remarkably, there is a notable alignment between these biological processes and the molecular functions involving the differentially expressed genes in IR (Figure 4C).

3.4. Validation of expression of the lncRNAs HOTAIR, LINCMD1 and PWRN2

The lncRNAs HOTAIR, LINCMD1, and PWRN2 were selected based on their significant upregulation observed in the microarray data analysis of Huh7-IR cells compared to Huh7-Ctrl cells (logFC ≥ 1.5; p-adjusted ≤ 0.05, to minimize false positives). Additionally, criteria for their selection included: 1) their association with key genes involved in metabolic and insulin signaling pathways, such as interleukins, SIRT1, AKT2, and AMPK; 2) their relationship with genes located near loci encoding interleukins, such as IL-17A and IL-17F; and 3) their report, in previously published original articles, of their relationship with metabolic or inflammatory processes associated with IR.

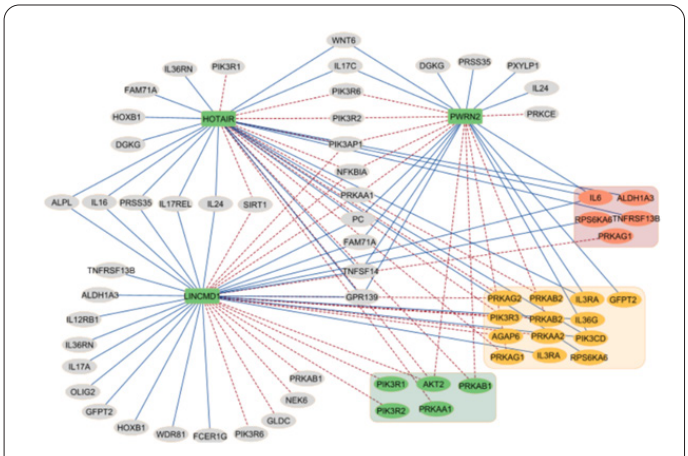

Fig. 3. lncRNA-mRNA coexpression network. Green rectangles represent lncRNAs, and circles represent protein-coding genes. Blue lines indicate positive correlations, while red dashed lines indicate negative correlations. Genes grouped in red are associated with IR, genes in green are related to insulin signaling, and genes in yellow share relationships with both metabolic pathways.

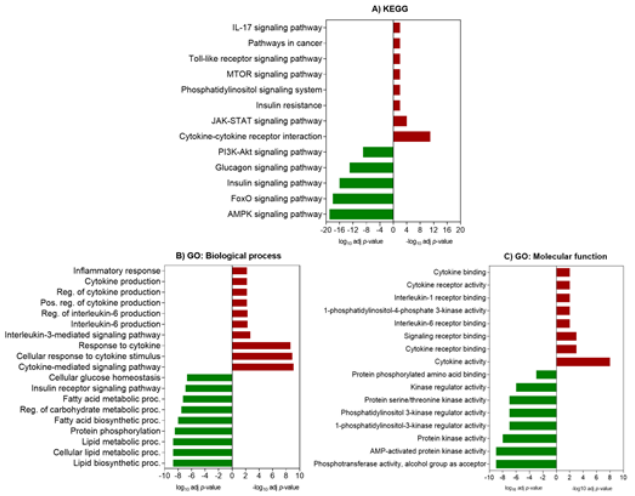

Fig. 4. Functional enrichment analysis of genes correlated with lncRNAs. A) KEEG pathway analysis: Upregulated genes (red) are associated with inflammatory pathways including JAK-STAT, IL-17, and TLR signaling. In contrast, downregulated genes (green) are linked to metabolic pathways, such as PI3K-Akt, AMPK, and insulin signaling. B) Gene ontology (GO) biological processes: Upregulated genes are enriched in processes related to cytokine production and response, whereas downregulated genes participate in lipid and carbohydrate metabolism. C) GO molecular functions: Upregulated genes exhibit cytokine activity, while downregulated genes demonstrate enzymatic activities, including kinases.

The expression of the three selected lncRNAs was assessed using RT-qPCR. Where HOTAIR expression was found to be 3.7-fold higher in Huh7-IR cells compared to Huh7-Ctrl cells ($p < 0.001$). Notably, treatment with metformin significantly reduced HOTAIR expression by 4.3-fold in Huh7-IR+Metf cells compared to Huh7-IR cells ($p < 0.001$). Similarly, LINCMD1 expression was 4.4-fold higher in Huh7-IR cells compared to Huh7-Ctrl cells but decreased significantly by 1.9-fold following metformin treatment ($p < 0.001$). On the other hand, PWRN2 expression increased by 1.5-fold in Huh7-IR cells compared to Huh7-Ctrl cells and decreased by 1.8-fold with metformin treatment (Figure 5A). These RT-qPCR results are congruent with the trends observed in the microarray analysis (data as described in Table 2) for all three lncRNAs (Figure 5B).

4. Discussion

In recent years, research has increasingly focused on the relationship between lncRNAs and metabolic diseases, not only exploring their expression but also their roles in molecular and metabolic processes linked to these conditions. Our group previously reviewed lncRNAs implicated in insulin resistance, highlighting MALAT1, H19, MEG, MIAT, and HOTAIR [3]. A recent review identified 33 lncRNAs involved in lipid metabolism and cardiovascular disease risk, including MALAT1, NEAT1, MIAT, H19, MEXIS, and GAS5 [13]. Similarly, several lncRNAs, such as SRA, Blnc1, MALAT1, Lfart, H19, and NEAT1, have

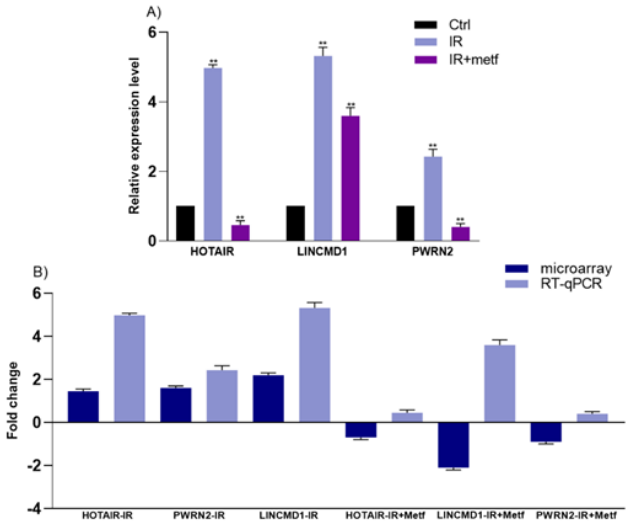

Fig. 5. Expressions of lncRNAs HOTAIR, LINCMD1 and PWRN2 obtained by RT-qPCR. A) Relative expressions of HOTAIR, LINCMD1, and PWRN2 in control (Ctrl), insulin-resistant (IR), and metformin-treated insulin-resistant (IR+Metf) cells. All three lncRNAs exhibit significant overexpression in IR cells, which is notably reduced following metformin treatment. Data are mean ± standard error (**$p < 0.001$). B) Comparison of relative expression changes (fold change) as determined by microarray analysis and RT-qPCR, demonstrating concordance between the two methods.

been associated with non-alcoholic fatty liver disease [14]. Advancing our understanding of how lncRNAs contribute to metabolic diseases is crucial for identifying therapeutic targets, developing treatments, and improving diseases monitoring and prognosis.

In this study, we utilized the Huh7 cell line to analyze the differential expression of lncRNAs in IR and subsequent treatment with metformin. Huh7 cells internalize glucose via the GLUT-2 transporter and activate the insulin signaling pathway through AKT phosphorylation [15]. Previously, our working group established the IR model in Huh7 cells, where exposure of cells to 10^{-6} mol/L insulin reduced glucose uptake and glycogen synthesis, while increasing intracellular lipids. Furthermore, decreased expression of phosphorylated insulin receptor, AKT, and pAMPK was observed in Huh7-IR compared to Huh7-Ctrl, underscoring the utility of Huh7 cells for metabolic assays in IR [12]. Hepatic IR is characterized by increased adipogenesis, decreased β-oxidation, and elevated release of triglycerides into circulation. Reduced AKT activation leads to decreased glucose uptake, allowing FOXO1 to promote gluconeogenesis while inhibiting glucose consumption and GLUT-2 expression [1].

Patients with IR have been observed to exhibit elevated levels of apoB-48 and apoA-IV. Metformin reduces IR by activating AMP-activated protein kinase (AMPK) and stimulating glucagon-like peptide-1 (GLP-1), which inhibits the production of apoB-48, apoA-IV, and triglycerides (TG). Additionally, metformin inhibits complex I of the mitochondrial respiratory chain, reducing ATP levels and increasing AMP levels, thereby favoring AMPK activation. This activation inhibits FOXO1 and suppresses SREBP-1c, leading to increase β-oxidation, reduced lipogenesis and TG levels [16]. The primary hypoglycemic effect of metformin is attributed to AMPK pathway activation. However, alternative mechanisms have also been proposed, including the enhancement of cellular redox potential, inhibition of complex IV in the mitochondrial respiratory chain, and binding to presenilin enhancer 2 (PEN2). These mechanisms inhibit v-ATPase, activate AMPK from lysosomes, inhibit acetyl-CoA carboxylase (ACC), and increase GLP-1 secretion, all of which contribute to reduced lipid accumulation and lower blood glucose levels [11]. Elevated TG levels and reduced glucose consumption have been observed in cells with IR. In contrast, treatment with metformin has been shown to decrease TG levels and increase glucose uptake, as demonstrated in studies involving HepG2 cells [20] and Huh7 cells [12] with IR.

The association of lncRNAs with IR, along with their molecular mechanisms and involvement in metabolic pathways, has been documented [3]. Our study identified 109 upregulated and 127 downregulated lncRNAs in IR, while with metformin treatment, the 109 lncRNAs that were upregulated in IR cells were found to be downregulated. Furthermore, among the lncRNAs that were downregulated in IR cells, only 60 of them were upregulated with metformin. Especially, the lncRNAs HOTAIR, LINCMD1, and PWRN2 were found to be upregulated in IR cells and were related to nearby genes involved in the insulin signaling pathway. HOTAIR is implicated in hepatic IR by potentially inhibiting AKT activation, thereby increasing hepatic glucose production through the upregulation of gluconeogenic enzymes, such as glucose-6-phosphate dehydrogenase and phosphoenolpyruvate carboxykinase. Studies have demonstrated that in HepG2 cells exposed to elevated levels of TNFα, HOTAIR expression increases. Additionally, HOTAIR inhibits SIRT1, a key regulator of hepatic insulin sensitivity. This negative regulation of SIRT1 by HOTAIR may disrupt the expression of genes encoding effector proteins in the insulin signaling pathway, contributing to elevated blood glucose levels, hepatic fatty acids accumulation, and IR [9]. High levels of HOTAIR in patients with type 2 diabetes (T2D) have been shown to help discriminate uncontrolled comorbidities related to the disease, suggesting its potential use as a biomarker for prognosis or early diagnosis [10].

In our study, we observed a reduced expression of the SIRT1 gene in Huh7-IR cells. However, treatment with metformin upregulated SIRT1 expression. Metformin is known to increase SIRT1 levels through AMPK activation. SIRT1 plays a pivotal role in lipid metabolism by deacetylating FOXO1, thereby preventing lipid accumulation in liver and adipocytes. Additionally, SIRT1 is believed to positively regulate AMPK signaling, likely through liver kinase B1 (LKB1) [18]. Based on our findings, we propose that SIRT1 also modulates the expression of genes encoding the catalytic subunit PRKAA and the non-catalytic subunits beta and gamma (PRKAB and PRKAG) of AMPK, along with the kinase responsible for AMPK activation, serine/threonine kinase 11 (STK11). Furthermore, SIRT1 interacts with the p85 adapter subunit of PI3K, enhancing PKB/AKT phosphorylation and promoting downstream insulin signaling [19]. Based on our results and interaction network analyses, we suggest that SIRT1 may modulate the expression of genes encoding AMPK subunits and key proteins such as PI3K/AKT2 in the insulin signaling pathway. Notably, these genes displayed similar expression patterns in both Huh7-IR and Huh7-IR+Metf cells. Our findings therefore suggest that HOTAIR negatively

regulates SIRT1, resulting in the dysregulation of multiple proteins involved in insulin signaling.

On the other hand, HOTAIR plays a pivotal role in the inflammatory response. Recent studies have shown that overexpression of HOTAIR inhibits the NFκB inhibitor alpha (IkBα), enabling NFκB to translocate to the nucleus and drive the transcription of proinflammatory cytokines [20]. Additionally, elevated HOTAIR expression has been linked to diabetes and its comorbidities. For example, in diabetic nephropathy, upregulation of HOTAIR has been observed in the podocytes of diabetic mice, with NFκB as a mediator [21]. In T2D, an intriguing relationship has been identified between HOTAIR upregulation, increased levels of proinflammatory cytokines, and decreased expression of SIRT1 [22]. Based on these findings and interaction network analyses, we infer that HOTAIR contributes to hepatic IR by downregulating SIRT1, upregulating NFκB, and promoting the secretion of proinflammatory cytokines associated with IR development.

Our analysis revealed that the LINCMD1 gene is located near the loci encoding IL17A, a key player in immune responses. IL17A-IL17F haplotypes have been identified as risk factors for the development of hip and knee osteoarthritis [23]. Moreover, IL17A has been shown to promote the production of inflammatory cytokines, including IL1β, IL6, and TNFα. Additionally, it activates mitogen-activated protein kinases and the NFκB inhibitor, ultimately promoting NFκB activation [24]. These observations suggest that LINCMD1 may influence the molecular effects mediated by IL17A and IL17F [29]. Specifically, we found that IL17A expression increased in Huh7-IR cells (logFC = 0.9), but significantly decreased in Huh7-IR+Metf cells.

Recently, in a lipopolysaccharide-treated retinal pigmented epithelium cell line, PWRN2 overexpression was found to promote NFκB activation by interacting with its inhibitor (NFKBIA or NFKBI), thereby enhancing NFκB signaling and increasing proinflammatory cytokine production. Conversely, downregulation of PWRN2 inhibited NFκB activation [25]. Therefore, we propose that PWRN2 may play a role in IR through its ability to activate NFκB, a key regulator of inflammation.

Furthermore, we identified differential gene expression in IR and found that genes associated with the selected lncRNAs are involved in IL-17, AMPK, and TLR signaling pathways, as well as IR. Our gene interaction network analysis suggests that inflammatory effector molecules play a crucial role in the development of IR development. Previous studies have shown that IL-1β, IL-6, TNF-α, and its transcription factor NFκB, contribute to IR by inhibiting INSR and insulin receptor substrate 1 (IRS1) phosphorylation through JAK/STAT pathway activation, which induces the expression of suppressor of cytokine signaling 3 (SOCS-3), potentially blocking insulin signaling [1, 26].

The critical role of lipids in the development of IR has also been well-documented. In our study, we observed abundant lipid droplets in Huh7-IR cells, supported by elevated TG levels. Fatty acids are known to trigger inflammation through both TLR-dependent and independent mechanisms, particularly by activating TLR4, which initiates NFκB-mediated transcription of inflammatory cytokines. These cytokines, in turn, activate kinases such as JNK and PKC, leading to the phosphorylation of serine residues on INSR, thereby impairing the insulin signaling pathway [2, 27].

As previously mentioned, HOTAIR negatively regulates SIRT1, a key modulator of hepatic insulin sensitivity. Additionally, the three lncRNAs –HOTAIR, LINCMD1, and PWRN2– contribute to the inflammatory response by enhancing the activation of NFκB, a molecule that promotes IR by inhibiting INSR and IRS1 [1, 26]. Further research is required to evaluate the role of the hepatic inflammatory response associated with the overexpression of HOTAIR, LINCMD1, and PWRN2 and its impact on the development of insulin resistance. Our findings show that metformin significantly modulates the expression of lncRNAs, including LINCMD1, PWRN2, and HOTAIR, as well as mRNAs associated with IR that are upregulated under IR conditions. Metformin achieves this by activating AMPK, which in turn enhances the activity of several epigenetic modifying enzymes, such as histone acetyltransferases, class II histone deacetylases like SIRT1, and DNA methyltransferases [28]. Furthermore, differential DNA methylation has been observed in individuals with T2D treated with metformin, with reports of 36 hypermethylated and 21 hypomethylated lncRNAs, linking hypermethylated lncRNAs to diabetic peripheral neuropathy [29]. Another study demonstrated that metformin downregulates the expression of HOTAIR and epithelial-mesenchymal transition markers, while also affecting the methylation of CpG islands within the HOTAIR promoter [30].

Our results have promising clinical implications. Monitoring these lncRNAs in insulin-resistant patients could serve as valuable biomarkers for evaluating treatment efficacy, such as with metformin, and could aid in personalizing therapies and tracking disease progression. Moreover, nucleic acid-based therapies, including antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and microRNAs (miRNAs), offer the potential to directly target mRNAs and noncoding RNAs (ncRNAs) [31], including lncRNAs [16]. In the future, siRNAs could be employed to reduce the expression of HOTAIR, LINCMD1, or PWRN2, thereby improving insulin sensitivity. Furthermore, specific patient groups, such as those with early-stage T2D, obesity-related IR, or resistance to conventional treatments, may benefit most from these innovative strategies. Future research should prioritize these populations to facilitate the clinical translation of these findings.

The overexpression of HOTAIR, LINCMD1, and PWRN2 in insulin-resistant Huh7 cells, and their reduction with metformin, suggests these lncRNAs play a role in the metabolic dysregulation of insulin resistance. While these findings align with prior studies on lncRNA regulation of glucose metabolism, limitations include the use of Huh7 cells, which do not fully replicate in vivo complexity or systemic interactions. Future research should validate these results in animal models or clinical samples and explore varying metformin treatments to better understand their therapeutic potential.

5. Conclusion

We conclude that the deregulation of lncRNAs, such as HOTAIR, LINCMD1, and PWRN2, contributes to hepatic insulin resistance by affecting inflammation and insulin signaling. Metformin mitigates these effects by reducing lncRNA expression and activating AMPK, which enhances glucose uptake, decreases lipid accumulation,

and restores SIRT1 expression. These findings underscore the therapeutic potential of targeting lncRNAs and epigenetic pathways in insulin resistance and related metabolic disorders. Future research should focus on the molecular interplay between lncRNAs, SIRT1, and key signaling pathways to develop targeted interventions for IR, T2D, and their complications.

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

Consent for publications
The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Conception and design: EFA, MC, MARV. Acquisition of data: VATF, YEM, CALV, JHGZ. Analysis and interpretation of data: MR, LDCAR, JACP, VATF, YEM. Writing of the manuscript: All authors contributed to drafting and revising the manuscript.

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References

1. Ziolkowska S, Binienda A, Jabłkowski M, Szemraj J, Czarny P (2021) The Interplay between Insulin Resistance, Inflammation, Oxidative Stress, Base Excision Repair and Metabolic Syndrome in Nonalcoholic Fatty Liver Disease. Int J Mol Sci 22:11128. https://doi.org/10.3390/ijms222011128.
2. Yaribeygi H, Maleki M, Sathyapalan T, Jamialahmadi T, Sahebkar A (2021) Pathophysiology of Physical Inactivity-Dependent Insulin Resistance: A Theoretical Mechanistic Review Emphasizing Clinical Evidence. J Diabetes Res 7796727. https://doi.org/10.1155/2021/7796727.
3. Tello-Flores VA, Beltrán-Anaya FO, Ramírez-Vargas MA, Esteban-Casales BE, Navarro-Tito N, Alarcón-Romero LDC, et al (2021) Role of Long Non-Coding RNAs and the Molecular Mechanisms Involved in Insulin Resistance. Int J Mol Sci 22:7256. https://doi.org/10.3390/ijms22147256.
4. Alsaedy HK, Mirzaei AR, Alhashimi RAH (2022) Investigating the structure and function of Long Non-Coding RNA (LncRNA) and its role in cancer. Cell Mol Biomed Rep 2(4): 245-253. https://doi.org/10.55705/cmbr.2022.360799.1062.
5. Yan C, Chen J, Chen N (2016) Long noncoding RNA MALAT1 promotes hepatic steatosis and insulin resistance by increasing nuclear SREBP-1c protein stability. Sci Rep 6:22640. https://doi.org/10.1038/srep22640.
6. Chen J, Ke S, Zhong L, Wu J, Tseng A, Morpurgo B, et al (2018) Long noncoding RNA MALAT1 regulates generation of reactive oxygen species and the insulin responses in male mice. Biochem Pharmacol 152:94-103. https://doi.org/10.1016/j.bcp.2018.03.019.
7. Zhu X, Wu YB, Zhou J, Kang DM (2016) Upregulation of lncRNA MEG3 promotes hepatic insulin resistance via increasing FoxO1 expression. Biochem Biophys Res Commun 469:319-25. https://doi.org/10.1016/j.bbrc.2015.11.048.
8. Chen DL, Shen DY, Han CK, Tian Y (2019) LncRNA MEG3 aggravates palmitate-induced insulin resistance by regulating miR-185-5p/Egr2 axis in hepatic cells. Eur Rev Med Pharmacol Sci 23:5456-5467. https://doi.org/10.26355/eurrev_201906_18215.
9. Li M, Guo Y, Wang XJ, Duan BH, Li L (2018) HOTAIR participates in hepatic insulin resistance via regulating SIRT1. Eur Rev Med Pharmacol Sci 22:7883-7890. https://doi.org/10.26355/eurrev_201811_16414.
10. Niknam N, Nikooei S, Ghasemi H, Zadian SS, Goudarzi K, Ahmadi SM, et al (2023) Circulating Levels of HOTAIR- lncRNA Are Associated with Disease Progression and Clinical Parameters in Type 2 Diabetes Patients. Rep Biochem Mol Biol 12:448-457. https://doi.org/10.61186/rbmb.12.3.448.
11. Foretz M, Guigas B, Viollet B (2023) Metformin: update on mechanisms of action and repurposing potential. Nat Rev Endocrinol 19:460-476. https://doi.org/10.1038/s41574-023-00833-4.
12. Villalva-Pérez JM, Ramírez-Vargas MA, Serafín-Fabían JI, Ramírez M, Elena Moreno-Godínez M, Espinoza-Rojo M, et al (2020) Characterization of Huh7 cells after the induction of insulin resistance and post-treatment with metformin. Cytotechnology 72:499-511. https://doi.org/10.1007/s10616-020-00398-4.
13. Gluba-Sagr A, Franczyk B, Rysz-Górzyńska A, Olszewski R, Rysz J (2024) The Role of Selected lncRNAs in Lipid Metabolism and Cardiovascular Disease Risk. Int J Mol Sci 25:9244. https://doi.org/10.3390/ijms25179244.
14. Zailaie SA, Khoja BB, Siddiqui JJ, Mawardi MH, Heaphy E, Aljagthmi A, et al (2024) Investigating the Role of Non-Coding RNA in Non-Alcoholic Fatty Liver Disease. Noncoding RNA 10:10. https://doi.org/10.3390/ncrna10010010.
15. Gunn PJ, Green CJ, Pramfalk C, Hodson L (2017) In vitro cellular models of human hepatic fatty acid metabolism: differences between Huh7 and HepG2 cell lines in human and fetal bovine culturing serum. Physiol Rep 5:e13532. https://doi.org/10.14814/phy2.13532.
16. Garcia D, Shaw RJ (2017) AMPK: Mechanisms of Cellular Energy Sensing and Restoration of Metabolic Balance. Mol Cell 66:789-800. https://doi.org/10.1016/j.molcel.2017.05.032.
17. Yang Q, Zhu Z, Wang L, Xia H, Mao J, Wu J, et al (2019) The protective effect of silk fibroin on high glucose induced insulin resistance in HepG2 cells. Environ Toxicol Pharmacol 69:66-71. https://doi.org/10.1016/j.etap.2019.04.001.
18. Cantó C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, et al (2009) AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458:1056-60. https://doi.org/10.1038/nature07813.
19. Fröjdö S, Durand C, Molin L, Carey AL, El-Osta A, Kingwell BA, et al (2011) Phosphoinositide 3-kinase as a novel functional target for the regulation of the insulin signaling pathway by SIRT1. Mol Cell Endocrinol 335:166-76. https://doi.org/10.1016/j.mce.2011.01.008.
20. Wang Y, Yi K, Liu X, Tan Y, Jin W, Li Y, et al (2021) HOTAIR Up-Regulation Activates NF-κB to Induce Immunoescape in Gliomas. Front Immunol 12:785463. https://doi.org/10.3389/fimmu.2021.785463.

21. Majumder S, Hadden MJ, Thieme K, Batchu SN, Niveditha D, Chowdhury S, et al (2019) Dysregulated expression but redundant function of the long non-coding RNA HOTAIR in diabetic kidney disease. Diabetologia 62:2129-2142. https://doi.org/10.1007/s00125-019-4967-1.
22. Sathishkumar C, Prabu P, Mohan V, Balasubramanyam M (2018) Linking a role of lncRNAs (long non-coding RNAs) with insulin resistance, accelerated senescence, and inflammation in patients with type 2 diabetes. Hum Genomics 12:41. https://doi.org/10.1186/s40246-018-0173-3.
23. Eftedal R, Vrgoc G, Jotanovic Z, Dembic Z (2019) Alternative Interleukin 17A/F Locus Haplotypes are Associated With Increased Risk to Hip and Knee Osteoarthritis. J Orthop Res 37:1972-1978. https://doi.org/10.1002/jor.24334.
24. Chen J, Liu X, Zhong Y (2020) Interleukin-17A: The Key Cytokine in Neurodegenerative Diseases. Front Aging Neurosci 12:566922. https://doi.org/10.3389/fnagi.2020.566922.
25. Song H, Wang YH, Zhou HY, Cui KM (2022) Sulforaphane alleviates LPS-induced inflammatory injury in ARPE-19 cells by repressing the PWRN2/NF-kB pathway. Immunopharmacol Immunotoxicol 44:868-876. https://doi.org/10.1080/08923973.2022.2090954.
26. Heo YJ, Choi SE, Jeon JY, Han SJ, Kim DJ, Kang Y, et al (2019) Visfatin Induces Inflammation and Insulin Resistance via the NF-κB and STAT3 Signaling Pathways in Hepatocytes. J Diabetes Res 2019:4021623. https://doi.org/10.1155/2019/4021623.
27. Wu H, Ballantyne CM (2020) Metabolic Inflammation and Insulin Resistance in Obesity. Circ Res 126:1549-1564. https://doi.org/10.1161/circresaha.119.315896.
28. Bridgeman SC, Ellison GC, Melton PE, Newsholme P, Mamotte CDS (2018) Epigenetic effects of metformin: From molecular mechanisms to clinical implications. Diabetes Obes Metab 20:1553-1562. https://doi.org/10.1111/dom.13262.
29. Solomon WL, Hector SBE, Raghubeer S, Erasmus RT, Kengne AP, Matsha TE (2020) Genome-Wide DNA Methylation and LncRNA-Associated DNA Methylation in Metformin-Treated and -Untreated Diabetes. Epigenomes 4:19. https://doi.org/10.3390/epigenomes4030019.
30. Golshan M, Khaleghi S, Shafiee SM, Valaee S, Ghanei Z, Jamshidizad A, et al (2021) Metformin modulates oncogenic expression of HOTAIR gene via promoter methylation and reverses epithelial-mesenchymal transition in MDA-MB-231 cells. J Cell Biochem 122:385-393. https://doi.org/10.1002/jcb.29867.
31. Zhu Y, Zhu L, Wang X, Jin H (2022) RNA-based therapeutics: an overview and prospectus. Cell Death Dis 13:644. https://doi.org/10.1038/s41419-022-05075-2.