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Targeting Metabolic Syndrome Pathways: Carrot microRNAs As Potential Modulators

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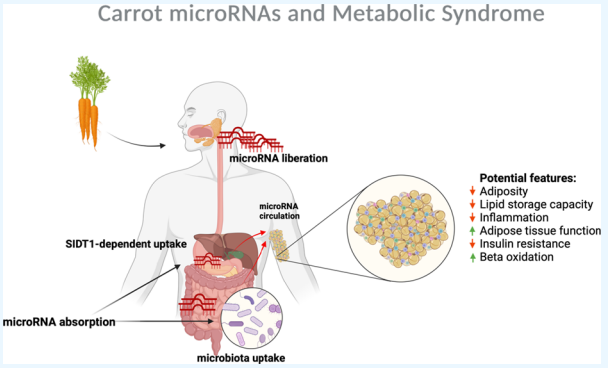
ABSTRACT: Metabolic syndrome is a condition characterized by metabolic alterations that culminate in chronic noncommunicable diseases of high morbidity and mortality, such as cardiovascular diseases, type 2 diabetes, nonalcoholic fatty liver disease, and colon cancer. Developing new therapeutic strategies with a multifactorial approach is important since current therapies focus on only one or two components of the metabolic syndrome. In this sense, plant-based gene regulation represents an innovative strategy to prevent or modulate human metabolic pathologies, including metabolic syndrome. Here, using a computational and systems biology approach, it was found that carrot microRNAs can modulate key BMPs/SMAD signaling members, C/EBPs, and KLFs involved in several aspects associated with metabolic syndrome, including the hsa04350:TGF-beta signaling pathway, hsa04931:insulin resistance, hsa04152:AMPK signaling pathway, hsa04933:AGE-RAGE signaling pathway in diabetic complications, hsa04010:MAPK signaling pathway, hsa04350:TGF-beta signaling pathway, hsa01522:endocrine resistance, and hsa04910:insulin signaling pathway. These data demonstrated the potential applications of carrot microRNAs as effective food-based therapeutics for obesity and associated metabolic diseases.



1. INTRODUCTION

Metabolic syndrome is characterized by clustering a set of metabolic disorders, including central obesity, dyslipidemia, hypertension, and insulin resistance.[1] The excessive accumulation of intra-abdominal fat is considered the central main risk factor for the development of metabolic alterations that culminate in the development of chronic noncommunicable diseases of high morbidity and mortality, such as cardiovascular diseases, type 2 diabetes, nonalcoholic fatty liver disease, colon cancer, etc.[2] The substantial increase in the prevalence of overweight and obesity considerably increases the predisposition to develop metabolic disorders at a very early age, which, according to the Organization for Economic Cooperation and Development (OECD) projections, will reduce life expectancy by more than four years in the next 30 years. There is no specific treatment for metabolic syndrome because of the lack of satisfactory controlled trial reports, and the currently available therapeutic strategies are usually focused on treating or preventing the individual components of metabolic syndrome.[3] Therefore, developing new approaches to manage this condition is extremely important, especially without long-term side effects.

Cross-kingdom gene regulation mediated by microRNAs has been suggested since the discovery of active plant microRNAs in the human organism, and the dietary-based uptake mechanism was elucidated.[4] The microRNAs are small, noncoding RNA molecules with approximately 18−25 nucleotides that regulate gene expression in many organisms, including humans. When a microRNA binds to a complementary sequence on the 3′-untranslated region of the mature mRNA, it promotes the degradation or translational repression of the target mRNA. This epigenetic regulation modulates several biological processes, such as embryonic development, cell proliferation, and differentiation.[5] Indeed, the miRNA-regulated epigenetic modifications likely act as determining factors in the development of pathologic conditions.

Although one of the major controversies of the cross-kingdom regulation of plant microRNAs is their functionality after dietary uptake, the preclinical and clinical studies have demonstrated the potential of fruit and vegetable microRNAs to target mammalian mRNAs, including humans.[6] For instance, using a bioinformatics approach, Olmi et al. examined the data of more than 380 experiments produced by 5 different next-generation sequencing projects; they revealed the presence of 350 circulating plant microRNAs in human blood across the analyzed data set.[7] Another study of ICR

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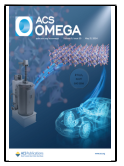

mice fed with *Brassica oleracea* RNA reported that exogenous plant microRNAs were present in the sera, feces, and tissues. Interestingly, miR-172, the most highly enriched microRNA in *B. oleracea*, was found in the stomach, intestine, serum, blood, spleen, liver, kidney, and feces of mice, and the amount of miR-172 that survived the passage through the gastrointestinal tract achieved a maximum of 4.5%.[8] A similar study demonstrated that dietary maize microRNAs could cross the gastrointestinal tract and reach the bloodstream of pigs, and influence gene expression similarly to mammalian microRNAs.[9]

Although research regarding the microRNA absorption mechanisms is still scarce, some studies point out that the digestion of uptake plant microRNAs begins in the mouth by saliva RNases, where the food matrix plays a protective role by encapsulating the microRNAs and protecting them from degradation during chewing.[10] Furthermore, the plant microRNAs could be absorbed in the stomach through a SIDT1-dependent mechanism, a nematode homologue systemic RNA interference defective protein 1 (SID-1) responsible for the transport of exogenous double-stranded RNA (dsRNA) into the cytoplasm.[11] In addition, plant microRNAs can be contained within exosome-like nanoparticles, protecting them from RNase degradation in the mouth. According to a report from a study of mice fed with ginger exosome-like nanoparticles, the microRNAs contained in the exosome-like nanoparticles reached the large intestine and were absorbed by the intestinal microbiota, altering the microbiome. This microbiome modification induced by ginger microRNAs improved mouse colitis, showing therapeutic activity.[12]

The first cross-kingdom study reported by Zhang et al. in 2012 demonstrated that the rice miR168a binds to the human/mouse low-density lipoprotein receptor adapter protein 1 mRNA, inhibiting its liver expression and consequently decreasing the LDL cholesterol plasma removal, demonstrating for the first time that plant microRNA influences the mammalian cholesterol transport.[13]

Likewise, miR159, a common plant microRNA found in *Arabidopsis thaliana*, *Glycine max*, and broccoli, can be detected in sera and tumor tissues after dietary intake. A study conducted by Chin et al. demonstrated that the soybean miR159 targeted the human *TCF7*, a transcription factor involved in the Wnt signaling and highly activated in breast cancer cells. Interestingly, when cancer cells were transfected or animals were fed with the synthetic miR159 mimic, the cancer cell growth was suppressed, accompanied by a significant reduction in *TCF7* and *MYC* expression, indicating an antitumoral effect mediated by this particular microRNA.[14] Recent studies of medicinal plant miRNAs using computational approaches have also predicted putative targets for cross-kingdom regulation for human disease. For instance, in *Ocimum basilicum*, the miR160, 414, and 869.1 were found to modulate human malignancy-related genes responsible for the crosstalk between various signal transduction pathways that eventually lead to activation of carcinogenic cascade.[15] Gadhavi et al. identified a set of 12 microRNAs in *Bacopa monnieri* that potentially target genes such as *TRAF2*, *CAV1*, *KLF6*, *CFLAR*, *PIK3R3*, *IL1B*, *HIF3A*, *ITGA4*, a set of genes with a significant role in the cancer pathway.[16] These findings proposed a cross-kingdom regulatory effect of plant microRNAs at the post-transcriptional level that could modulate pathways associated with human diseases. Due to this cross-kingdom regulation, the research regarding the nutraceutical properties of dietary microRNAs is a frontier research topic, and the use of mature sequences of plant miRNAs through specific enriched diets and food supplements represents a promising alternative to modulate pathologic conditions.[17]

In the metabolic syndrome context, the carrot (*Daucus carota* L.) has an essential role in preventing this condition because it provides significant amounts of different nutraceuticals, including carotenoids, dietary fiber, and phenolics, that exhibit an antidiabetic, antiobesity, anti-inflammatory, and cardioprotective potential.[18] Although the effects of the carrot microRNAs and how they could regulate metabolic processes to exert a therapeutic function remain unknown, their therapeutic value is currently being researched for various dysfunctions, and the potential of these microRNAs may soon be valued as a possible approach in plant-based drugs to manage several diseases. Thus, this study aims to predict the human target genes that can be plausibly modulated by carrot microRNAs using a computational and system biology approach. Furthermore, a functional annotation and pathway mapping analysis of the identified specific gene targets was performed.

2. MATERIALS AND METHODS

2.1. Data Retrieval. Analyzing the literature from the PubMed and Scopus databases on small RNA sequencing on *Daucus carota* L., a study published in 2019 by Bhan et al. reported the identification and expression profiling of the orange-red carrot microRNA.[19] From this study, 102 microRNA sequences were recovered and used as a reference to predict the target genes in a *Homo sapiens* transcriptome and identify their functional properties and signaling and disease-related associations. The additional literature was employed to complement the proposed involvement of the predicted target genes in specific signaling pathways related to the metabolic syndrome.

2.2. Prediction of Potential Human Targets. Human target genes were predicted using the psRNAtarget web server (2017 release), a tool for determining the reverse complementarity between small RNA and target-site of target transcript and assessing the target accessibility by computing unpaired energy.[20] The psRNATarget web server has one of the best algorithms for target prediction, with 74% of precision and 62% of recall.[21] The mature carrot microRNA sequences were queried for human gene target prediction against the cDNA library of *Homo sapiens* transcripts available on the web server. The default setting was used in the V2 scoring schema; the maximum expectation was set to 5. Lower values provide more stringent prediction results but more potential target sequences might be missed. Considering 5 of expectation value is ideal to get acceptable targeting results, while expectation over 5 may give unreliable targeting results.[22] The penalty for the G:U pair was set to 0.5, the penalty for other mismatches was set to 1, and the penalty for the opening gap was set to 2. Moreover, the number of mismatches allowed in the seed region was set to 2 (mismatches of more than 2 in seed regions give rise to unreliable targeting results). Translation inhibition was set between 9 and 11 nucleotides, and the length for complementarity scoring size was set to 19. Unfortunately, the UPE, which indicates the accessibility-maximum energy required to unpair the RNA secondary structures, was not calculated in the V2 schema because this approach assumes a priori that this factor does not influence the miRNA-target sequence interaction.[23]

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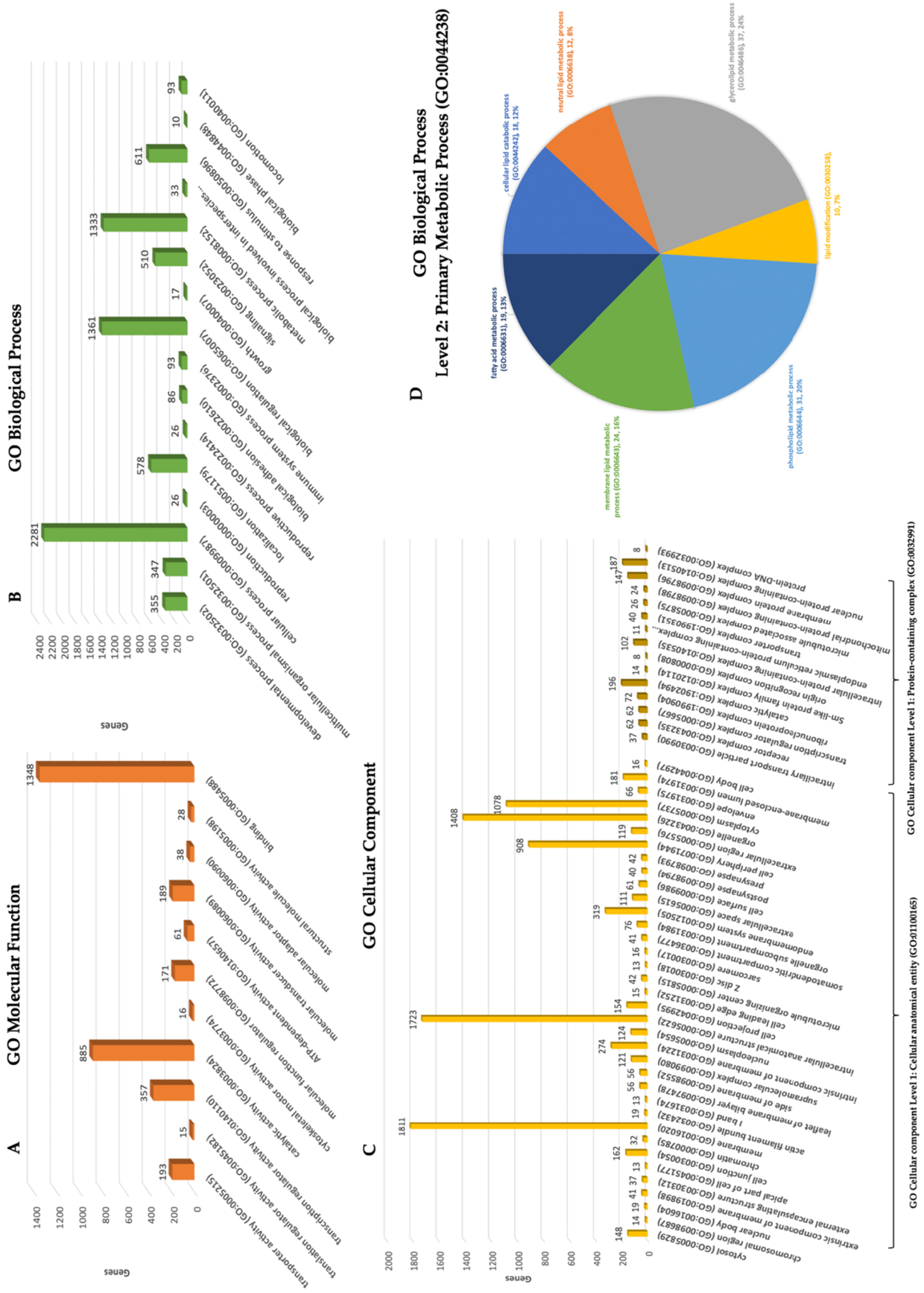

Figure 1. Functional Annotation of the predicted genes using PANTHER. All predicted target genes were categorized by gene ontology into the independent hierarchies of (A) molecular function, (B) biological processes, and (C) cellular components. (D) Subcategory within the metabolic process category of the biological process presented in B. All terms were considered significant considering at least eight hits and *p*-value <0.05.

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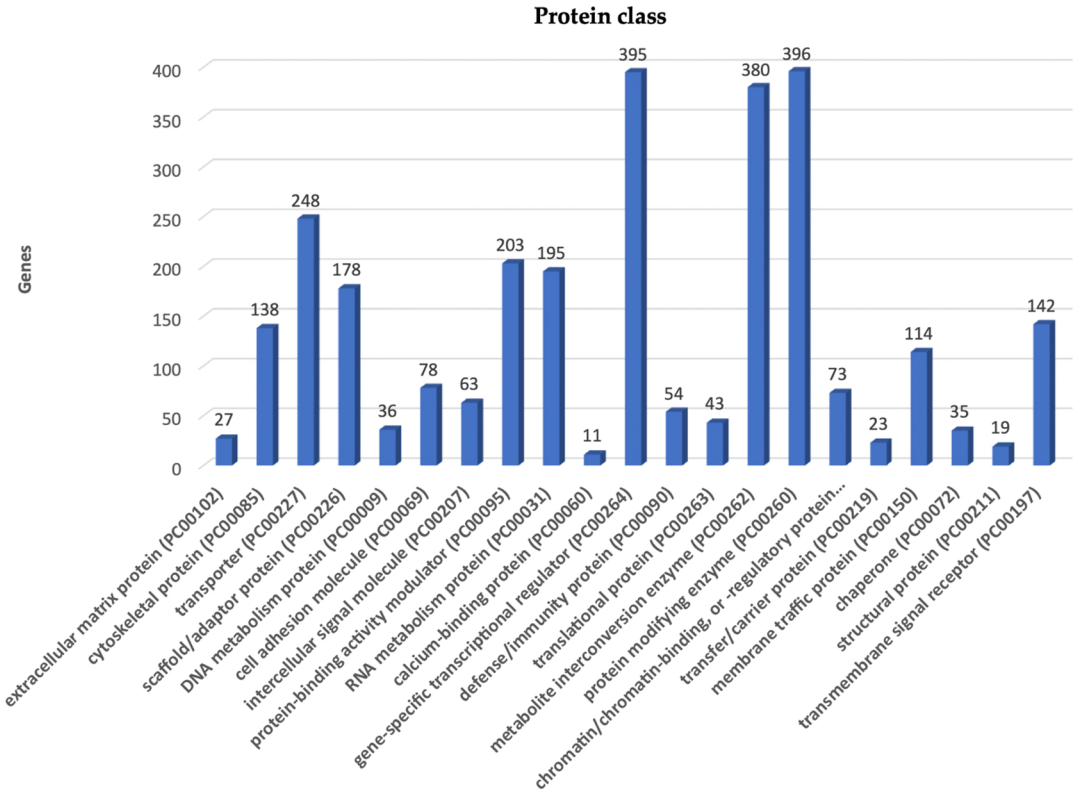

Figure 2. Protein class annotation using PANTHER. All predicted genes were categorized by the function of their protein functional class. All terms were considered significant considering at least eight hits and p-value <0.05.

2.3. Functional Annotation Analysis and Pathway Analysis. Identifying the properties of predicted target genes is essential to understanding the complex network of regulatory pathways modulated by carrot microRNAs and their role in biological processes. In this sense, a functional enrichment analysis for the predicted target genes was performed using the Protein Analysis THrough Evolutionary Relationships (PANTHER; v17.0) classification system.[24] The predicted target genes were categorized by gene ontology (GO) into three independent hierarchies, namely, biological processes, cellular components, and molecular functions. Also, a protein class categorization was made. The terms were considered statistically significant with a threshold p-value of ≤0.05. The DAVID (Database for annotation, visualization, and Integrated Discovery)[25] was used to map the predicted gene targets in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and REACTOME pathways databases using the database default settings. A pathway was significantly enriched only if it passed the count threshold of 10 genes per annotation term and presented a p-value ≤0.05. To identify key genes from all the predicted targets, the STRING database was used to decipher the protein−protein Interaction network and infer the role of noteworthy, predicted genes in different cell processes.[26]

2.4. Gene−Disease Associations. The predicted miRNA target genes may be involved in developing and progressing several diseases including metabolic syndrome. A gene-disease association analysis was performed based on the predicted target gene list to identify the disease types associated with these predicted target genes. All genes were mapped against the online DisGeNET platform *v7.0* using default settings to understand the associations wherein one gene may be involved in several diseases and vice versa. The DisGeNET is a curated database of gene-disease associations integrated from the most popular databases and the mining of disease-associated genes from the literature.[27,28]

3. RESULTS AND DISCUSSION

3.1. Carrot microRNAs Can Target Human Genes. The discovery of still active plant microRNAs in the human organism and the dietary-based uptake suggest that plant-based foods can play a role in modulating host gene expression.[4] Therefore, these molecules represent an innovative strategy to prevent or modulate human metabolic pathologies. Previous studies have evaluated the effects of carrot microRNAs in regulating physiological processes in the plant.[19,29] However, the trans-kingdom regulation of carrot microRNAs remains unknown. In this study, the 102 carrot microRNA sequences previously reported were used to predict potential targets against a library of *Homo sapiens* transcripts.

A total of 3947 potential target genes of the 102 carrot microRNAs, were predicted using the psRNATarget Web server. The results in Table S1 show that most targets indicated the inhibition function as "cleavage," and few were classified in the "translation" category. The psRNATarget tool validates miRNA−miRNAs interactions, estimating the mRNA target availability and energy needed to unwind the secondary structure throughout the target site to calculate target accessibility. Although several tools for target prediction are available, only a few systems, including the psRNATarget, are designed to estimate a proper cross-kingdom interaction.[23] According to the psRNATarget predictions performed in this study (Table S2), the gma-miR156n, sly miR395b, gra-miR7494c, osa-miR156l-3p, bgy-miR156 and carrot-m0026-5p are the microRNAs with more possible targets, potentially

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modulating 158, 148, 146, 134, 126, and 121 predicted target genes, respectively. Interestingly, the overall gene target prediction accounts for 84.31% of the summited carrot microRNAs. A total of 16 microRNAs, including ppt-miR533a-5p, stu-miR167c-3p, pgi-miR2118, ath-miR159b-3p, stu-miR6024-5p, ath-miR8175, hvu-miR6188, hbr-miR6173, bra-miR164e-5p, ath-miR319b, gma-miR166k, smo-miR408, ath-miR403-3p, sbi-miR164c, stu-miR6149-5p, and gma-miR6300, did not get targets.

3.2. Carrot microRNAs Could Influence Human Lipid Metabolism. The GO annotation provides knowledge regarding a particular protein's molecular function, processes, and location. These data can be used to predict the function and biological roles of new gene products, providing a link between biological knowledge and gene expression profiles.[30]

The results of the Gene Ontology annotation (Figure 1) indicate that the primary molecular function (of the predicted target genes is binding (GO:0005488), followed by catalytic activity (GO:0003824), transcription regulatory activity (GO:0140110) and transporter activity (GO:0005215), representing 33.70, 22.1, 8.90, and 4.80% of all annotated genes, respectively (Figure 1A).

In the case of biological processes (Figure 1B), the main categories were cellular process (GO:0009987), biological regulation (GO:0065007), metabolic process (GO:0008152), and response to stimulus (GO:0050896) with 57.0, 34.0, 33.30, and 15.30% of the genes, respectively.

On the other hand, for the cellular component category (Figure 1C), it is observed that the majority of the predicted target genes located in a cellular anatomical entity GO:01100165 are mainly in the membrane (GO:0016020), intracellular anatomical structure (GO:0005622), organelle (GO:0043226), cytoplasm (GO:0005737), and cell periphery (GO:0071944). At the same time, at the protein-containing complex level (GO:0032991), the predicted target genes are found in the catalytic complex (GO:1902494), nuclear-protein-containing complex (GO:0140513), membrane protein complex (GO:0098796), and intracellular protein-containing complex (GO:0140535).

Analyzing the metabolic process (GO:0008152) category (Figure 1D) at Level 2: Primary metabolic process (GO:0044238), it was found that 77 genes regulate processes involved in lipid metabolism, such as cellular lipid catabolic process (GO:0044242), neutral lipid metabolic process (GO:0006638), glycerolipid metabolic process (GO:0046486), lipid modification (GO:0030258), phospholipid metabolic process (GO:0006644), membrane lipid metabolic process (GO:0006643), and fatty acid metabolic process (GO:0006631).

Our analysis underscores the potential of carrot microRNAs in modulating genes implicated in insulin receptor signaling, glucose transport, and lipid metabolism pathways, which are crucial for maintaining insulin sensitivity. This suggests a novel approach to mitigating insulin resistance, a key component of metabolic syndrome, highlighting the mechanistic links between dietary microRNAs and the regulation of metabolic pathways associated with insulin resistance.

Regarding the protein class category (Figure 2), it is observed that the products of the predicted gene targets are mainly protein modifying enzyme (PC00260), gene-specific transcriptional regulator (PC00264), metabolite interconversion enzyme (PC00262), transporter (PC00227), protein-binding activity modulator (PC00095) and membrane trafficking protein (PC00150). Interestingly, PANTHER did not annotate 46.10% of the predicted target genes by molecular function, 38.80% by biological process, 40.2% by cellular component, and 28.5% by protein class. In summary, our findings suggest that carrot microRNAs have the potential to modulate key signaling pathways involved in metabolic syndrome, particularly those related to insulin resistance, underscoring the importance of dietary microRNAs as potential modulators of human health. This highlights the need for further experimental studies to explore their therapeutic implications.

In the neutral lipid metabolic (GO:0006638) process, there are involved genes such as *LPIN3*, *DGKB*, *GK5*, *DGKG*, *PNPLA2*, *PCK1*, *AWAT1*, *ABHD2*, *DGKD*, *ABHD5*, *AWAT2*, and *DGAT1*. In the case of the fatty acid metabolic process, there are predicted genes such as *PTGS1*, *LPIN3*, *ACSL4*, *ACOX1 PTGIS*, *ACSF3*, *ELOVL5*, *DCAF5*, *BAAT*, *ELOVL1*, *PCK1*, *PTGES3*, *ACAT1*, *ACBD5*, *ABHD2*, *MCAT*, *ACOX3*, *WDTC1*, and *ACSL6*.

The psRNATarget prediction shows that the carrot-m0025-3p, zma-miR396g-5p, and carrot-m0029-3p possible targets the *ACSL6* and *ACSL4* genes, two members of the long-chain-fatty-acid-CoA ligases family. This ligase family catalyzes the thioesterification of long-chain fatty acids to their active form acyl-CoA for both synthesis of cellular lipids, and degradation via beta-oxidation.[31] Currently, there are five established ACSLs (1−5) expressed in different tissues and cellular locations depending on their functional role. In the case of *ACSL6* (formerly *ACSL2*), its enhanced expression drives acyl-CoA toward lipid synthesis.[32]

Results of Teodoro et al. show that *ACSL6* expression on skeletal muscle is promoted by an acute lipid ingestion state while fasting and exercise conditions induce downregulation of the *ACSL6* expression. The *ACSL6* knockdown decreased the accumulation of triglycerides (TAG), and lipid droplets, an increase in fatty acid content, p-AMPK, mitochondrial content, mitochondrial respiratory rates, and palmitate oxidation, and the expression of the transcriptional coactivator PGC-1α, suggesting promotion of lipid oxidation and enhanced mitochondrial respiration with the activation of the AMPK/PGC1-α pathway.[32] Jung and Bu revealed that ACSL6 acts on fatty acids derived from exogenous sources and via de novo synthesis. The knockdown of *ACSL6* decreased the partitioning of fatty acids into TAG and diglycerides (DAG) without altering total cellular phospholipids levels. In addition, glucose uptake is suppressed following the *ASCL6* knockdown.[33] Another study has shown that *ACSL6* expression is elevated in a rat model of nonalcoholic fatty liver disease (NAFLD), which may be associated with an increased lipid metabolism and liver cell apoptosis.[34]

In the case of *ACSL4*, it catalyzes the conversion of long-chain fatty acids to their active form acyl-CoA, preferentially using arachidonate and eicosapentaenoate as substrates.[31] Correlative studies suggested a possible role in promoting the hepatic TAG synthesis. For instance, Kudo et al. demonstrated that *ACSL4* suppression under high-fat dietary conditions may have attenuated the accumulation of triglycerides in the liver compared to wild-type mice under the same conditions.[35] Westerbacka et al. reported that *ACSL4* gene expression is upregulated in the livers of insulin-resistant human subjects with NAFLD.[36] Golej et al. found that *ACSL4* overexpression in human arterial smooth muscle cells resulted in a preferred increased synthesis of arachidonyl-CoA and in the incorpo-

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ration of arachidonic acid into phosphatidylethanolamine and phosphatidylinositol, significantly elevating the synthesis of TAG and DAG. In contrast, the *ACSL4* silencing attenuates the PGE_2 release.[31]

The presence of acyl-CoA thioesterases with different substrate preferences is likely to play an important role in regulating acyl-CoA levels available for incorporation into different cellular lipid pools. These data show that carrot-microRNA could influence processes related to cell energy homeostasis, modulating key aspects of immune response, lipid metabolism, and mitochondrial function.

3.3. Carrot microRNAs Potentially Modulate Metabolic Syndrome-Associated Pathways. In this study, the DAVID mapping of the predicted target genes in the KEGG signaling pathways (Figure 3) shows probably 77 signaling pathways. The hsa05200:Pathways in cancer, hsa04151:PI3K-Akt signaling pathway, and hsa04010:MAPK signaling pathway are the pathways with more genes involved with 138, 98, and 81 predicted genes, respectively. Among the significantly predicted genes that stand out for their involvement in signaling pathways related to cell survival, proliferation, and differentiation are the *KLF* transcription factors, *SMADs*, *BCL-2*, *CREB*, *Wnt*, *PRMDs*, *GDFs*, and *BMP* receptors, *CEBPs*, *PPARA*, and some growth factors such as *FGF*, *HGF*, *PDGF*, *IGF*, and *TGF-β*.

Predicted target genes involved in KEGG signaling pathways related to metabolic syndrome are highlighted in Table 1. The most representative are hsa04931:Insulin resistance, hsa04152:AMPK signaling pathway, hsa04933:AGE-RAGE signaling pathway in diabetic complications, hsa04010:MAPK signaling pathway, hsa04350:TGF-beta signaling pathway, hsa01522:Endocrine resistance, and hsa04910:Insulin signaling pathway.

Obesity is the central axis that increases the risk of multiple diseases, such as type 2 diabetes, insulin resistance, cardiovascular disease, hypertension, NAFLD, and certain cancer types.[37] Interestingly, here it was found that one of the signaling pathways with a high impact on metabolic syndrome is the hsa04350:TGF-beta signaling pathway. This signaling pathway plays diverse roles in appetite regulation, lipid metabolism, and glucose homeostasis. The transforming growth factor beta (TGFβ) superfamily members, including TGFβ, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, and nodal-related protein, control several aspects of adipogenesis, such as the regulation of white, brite and brown adipocyte differentiation and adipocyte metabolic and endocrine functions.[38]

In this study, it was found that carrot microRNAs target some components of BMP signaling (Figure 4). The psRNATarget prediction analysis shows that ath-miR169a-3p potentially targets *SMAD1*; osa-miR156l-3p and gra-miR7494c to *SMAD4*; sly miR395b and carrot-m0025-3p to *BMPR1A*; osa-miR156l-3p to *GDF6*; and carrot-m0026-5p and bgy-miR156 to *WWTR1*.

The BMP signaling stimulates adipogenesis by increasing the expression levels of PPARγ through the SMAD1/5/8-dependent mechanism. This signaling is mediated by activation of the heteromeric receptor complexes type I and type II; BMP2, BMP4, and GDF6 bind primarily to the type I receptor recruiting type II into the heteromeric signaling complex.

The phosphorylated BMP receptors bind and activate the SMAD1, 5, and or 8 (SMAD1/5/8) and then associate with SMAD4 to translocate to the nucleus via the nuclear transporter TAZ (WWTR1) and upregulate transcription of PPARγ.[38,39] In this sense, modifying this signaling pathway represents a good option for modulating early processes of

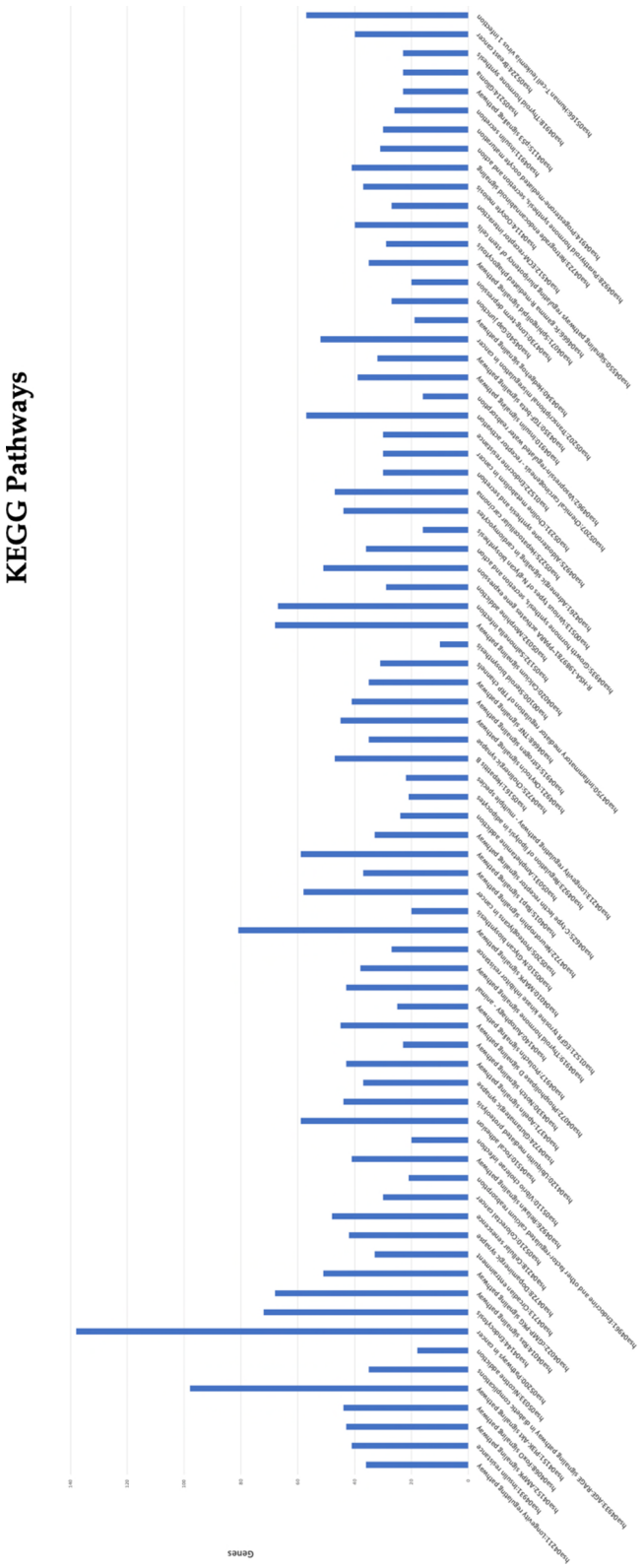

Figure 3. KEGG pathway mapping using DAVID. The predicted microRNA can be involved in 77 KEGG pathways (p-value <0.05). A predicted gene may exhibit more than one function; thus, it can be included in multiple signaling pathways.

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Table 1. Potential Carrot microRNAs to Modulate KEGG Signaling Pathways Involved in the Metabolic Syndrome[a]

KEGG pathway	predicted human target genes	carrot microRNA
hsa04910:insulin signaling pathway	*PYGB, SHC3, PRKAA2, IRS1, PDE3B, PRKAG1, PIK3R3, IRS2, CALML3, CALML4, PIK3CB, ELK1, FOXO1, SOCS2, MAPK9, GYS1, PRKAR2A, MKNK1, PHKG2, AKT3, PCK1, HRAS, EIF4E, SOCS4, PKLR, PDPK1, INSR, PHKB, TSC1, PRKAB1, G6PC2, PPP1R3C, PPP1R3B, RHEB, RPS6KB2, GRB2, RAF1, SOS1, RHOQ*	sly miR395b, zma-miR169l-5p, mtr-miR168a, osa-miR166e-3p, csi-miR166b, carrot-m0008-3p, ppt-miR390b
hsa04350:TGF-beta signaling pathway	*IGSF1, THBS1, THSD4, ACVR1C, PPP2R1B, PPP2R1A, SIN3A, PITX2, SMAD2, SMAD1, TGFB2, SMAD4, TGIF2, SMAD3, TGFB3, NOG, SMAD9, INHBA, RGMB, GDF6, ACVR2B, TGFBR1, BMP6, ACVR2A, RGMA, TGFBR2, ZFYVE16, ID1, RPS6KB2, FMOD, NBL1, BMPR1A*	carrot-m0032-5p, carrot-m0006-5p, carrot-m0002-3p, ath-miR169a-3p, osa-miR156l-3p, gra-miR7494c, aly miR398a-5p, ppt-miR390b
hsa01522:endocrine resistance	*NOTCH2, NOTCH3, SHC3, PIK3R3, ADCY1, PIK3CB, ADCY7, ADCY6, IGF1R, MAPK9, AKT3, HRAS, JAG2, MED1, JAG1, BIK, NCOA3, IGF1, FOS, ESR1, MAPK13, MAPK11, ADCY9, RPS6KB2, BCL2, GNAS, GRB2, RAF1, SOS1, TP53*	carrot-m0014-3p, carrot-m0051-5p, carrot-m0032-5p, carrot-m0009-3p, zma-miR172d-5p, ppt-miR319d-3p, mes-miR166i
hsa04919:thyroid hormone signaling pathway	*PFKFB2, NOTCH2, NOTCH3, THRB, SLC2A1, ATP2A2, PIK3R3, PIK3CB, MED12L, FOXO1, ACTB, SLC9A1, CASP9, RXRB, SIN3A, AKT3, ITGAV, PLCG1, SLC16A2, HRAS, WNT4, NCOA1, MED1, PRKCB, PDPK1, STAT1, NCOA3, DIO2, ATP1B1, ESR1, MED13L, KAT2B, PLCB3, KAT2A, RHEB, PLCB1, RAF1, TP53*	carrot-m0039-3p, mes-miR397, carrot-m0016-5p, ata-miR166b-5p, osa-miR166i-5p, zma-miR172d-5p, ata-miR172c-5p
hsa04010:MAPK signaling pathway	*FLT1, RASGRF2, RASGRF1, IL1RAP, ELK1, IGF1R, ELK4, FGF6, RPS6KA6, FGF7, RPS6KA2, AKT3, STMN1, KDR, MAP3K9, HRAS, MAP3K7, DUSP4, MAP2K4, DUSP5, MEF2C, DUSP3, PRKCB, PLA2G4E, HGF, CACNA2D2, FOS, MAPK8IP3, TGFBR1, TGFBR2, EREG, IL1A, CACNB3, MRAS, TRAF6, MAPKAPK2, MAPT, RAF1, SOS1, TP53, CSF1R, PDGFB, CACNA1A, CACNA1C, NLK, CACNA1E, RASGRP3, MAPK9, CACNG8, PPP3R1, PAK1, PPP3R2, ERBB3, ERBB4, MKNK1, GNA12, FGF20, CACNG2, MAP2K7, MAP4K3, FGF21, MAP3K2, NTRK2, MAP3K3, TGFB2, ANGPT2, GADD45B, ANGPT1, TGFB3, EGF, INSR, IGF2, IGF1, NFKB1, MAPK13, MAPK11, TAOK3, TAOK1, NF1, GRB2*	ata-miR172c-5p, bna-miR169k, ata-miR166b-5p, bdi-miR166e-3p, gra-miR7494c, nta-miR479a, vvi-miR171g, mdm-miR396a
hsa04931:insulin resistance	*PYGB, SLC27A1, PRKAA2, IRS1, PRKAG1, PTEN, SLC2A1, PIK3R3, IRS2, PIK3CB, FOXO1, MAPK9, GYS1, RPS6KA6, CREB3L3, RPS6KA2, AKT3, CREB3L2, MLX, CD36, PCK1, PPARGC1B, MLXIP, PRKCB, PDPK1, PRKCE, NR1H2, GFPT2, INSR, PRKCD, PRKAB1, NFKB1, MLXIPL, CREB3, G6PC2, CREB1, PPP1R3C, PPP1R3B, RPS6KB2, PPARA, OGT*	sly miR395b, zma-miR169l-5p, carrot-m0051-5p, gma-miR1511, carrot-m0017-3p, ata-miR172c-5p, mes-miR397
hsa04152:AMPK signaling pathway	*PFKFB2, PRKAA2, CAB39, CAB39L, IRS1, PRKAG1, PIK3R3, IRS2, PIK3CB, FOXO3, ADIPOR1, ELAVL1, FOXO1, CAMKK2, IGF1R, GYS1, EEF2K, PPP2R1B, CREB3L3, PPP2R1A, HNF4A, AKT3, CREB3L2, LEPR, CD36, PCK1, MAP3K7, RAB2A, PDPK1, INSR, SCDS, CIDEA, TSC1, PPP2R3A, IGF1, PPP2R5C, PRKAB1, CREB3, G6PC2, CREB1, SCD, RHEB, RPS6KB2*	aly miR319b-5p, carrot-m0051-5p, carrot-m0029-3p, ppe-miR396a, mtr-miR168a, gra-miR7494c, carrot-m0039-3p
hsa04933:AGE-RAGE signaling in diabetic complications	*PIK3R3, PIK3CB, FOXO1, MAPK9, AKT3, PIM1, PLCG1, HRAS, SMAD2, TGFB2, SMAD4, SMAD3, TGFB3, PRKCB, STAT1, PRKCE, PRKCD, CYBB, TGFBR1, NFKB1, TGFBR2, MAPK13, COL1A1, DIAPH1, IL1A, MAPK11, PLCB3, COL1A2, COL4A4, COL4A3, COL4A6, BCL2, COL4A5, NOX4, PLCB1*	ppt-miR477e, osa-miR166i-5p, cca-miR396a-3p, carrot-m0010-3p, nta-miR479a, osa-miR156l-3p, carrot-m0016-5p, nta-miR479a

[a]For more detailed information about the predicted target gene and microRNA, see Table S2.

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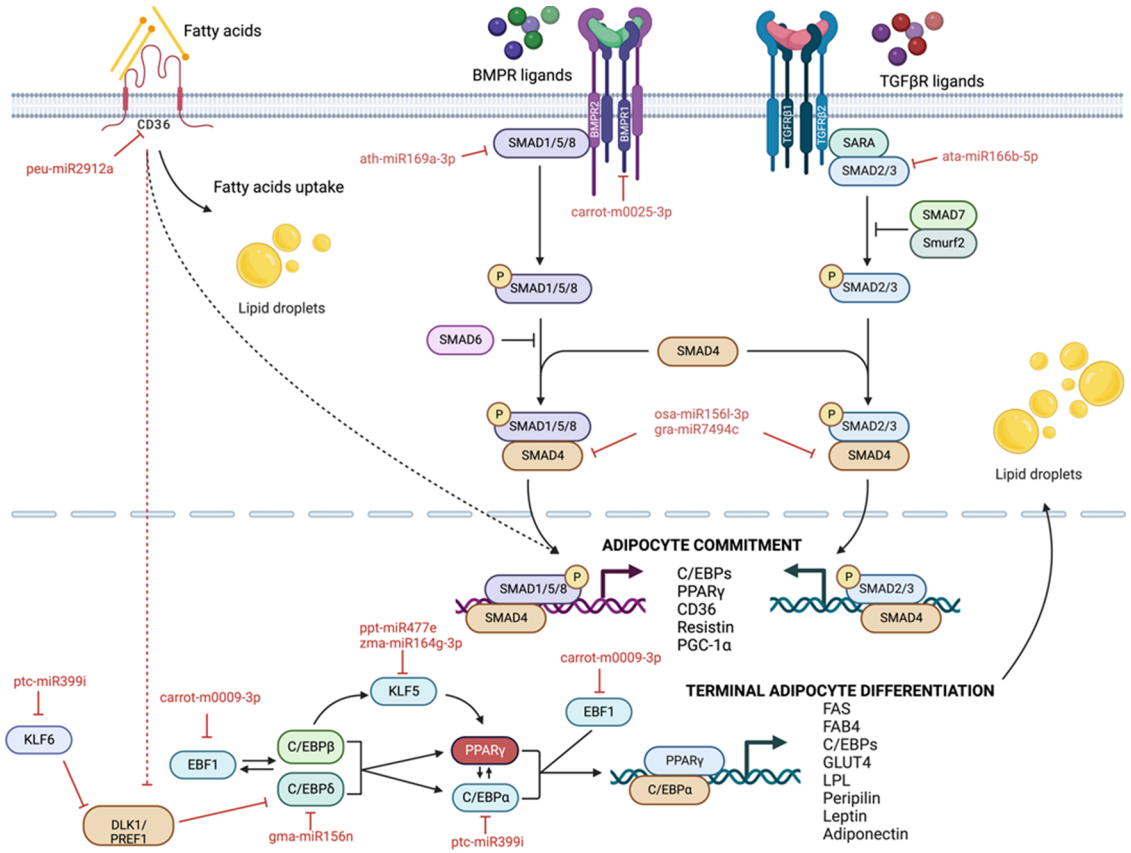

Figure 4. Potential effect of carrot microRNAs on adipogenesis. Carrot microRNAs target factors necessary for cellular determination, such as TGFβ/SMAD signaling members, C/EBPs, and KLFs. Therefore, it is possible to alter this early programming and modify fat accumulation, fatty transport, sensitivity to insulin, and chronic inflammation, among others. Created in BioRender.com.

obesity. For example, Wang et al. reported that *SMAD4* silencing reduced GDF6-induced adipocyte lineage commitment of C3H10T1/2 cells.[40] Similarly, Huang et al. reported that the knockdown of SMAD4 disrupts the BMP2/4-induced adipogenic commitment process in the same cellular model.[41] Long et al. demonstrated that inhibition of upstream components, such as BMPR1 promotes preadipocyte proliferation but inhibits their differentiation in a culture of bovine preadipocytes.[42]

In addition, the ata-miR166b-5p potentially targets *SMAD3*, a multifaceted regulator in adipose physiology and the pathogenesis of obesity and type 2 diabetes mediated by the TGF-β1/SMAD3 signaling.[43] In vivo studies have suggested that blocking the TGF-β1/SMAD3 signaling represents a holistic approach to treating metabolic syndrome factors since it may protect from obesity, diabetes, and hepatic steatosis. For instance, experiments in SMAD3 knockout mice revealed that *SMAD3* loss improves hypoglycemia and insulin resistance, reduces adiposity by inducing the white to brown adipocyte transdifferentiation, and prevents diet-induced obesity.[44]

Carrot microRNAs can modulate several aspects of the hsa04350:TGF-beta signaling pathway, mainly promoting adipogenesis by BMPs and TGF-β1 signaling. Using foods enriched with these microRNAs targeting SMAD signaling represents an interesting approach to modulating various factors associated with metabolic syndrome.

On the other hand, the results of the predicted target genes annotated in the REACTOME database with DAVID (Table 2) show that the main signaling pathways that can be associated with metabolic syndrome are R-HSA-400206—regulation of lipid metabolism by PPAR-alpha, R-HSA-9006936—signaling by TGFB family members, R-HSA-1489509—DAG and IP3 signaling, R-HSA-163685—integration of energy metabolism, and R-HSA-381340—transcriptional regulation of white adipocyte differentiation.

Analyzing the genes involved in R-HSA-381340-transcriptional regulation of white adipocyte differentiation pathway, notable genes were found such as *C/EBPα*, which is potentially modulated by ptc-miR399i, *C/EBPδ* by gma-miR156n; *KLF5* by ppt-miR477e and zma-miR164g-3p, *KLF6* by ptc-miR399i; *CD36* by peu-miR2912a and *EBF1* by carrot-m0009-3p (Figure 4; Table S2).

C/EBPα and C/EBPδ are representative members of the CCAAT/enhancer binding proteins family with a positive and critical role in the adipogenic program.[45] Studies in adipogenic cells have shown that after the differentiation induction of adipocytes, there is a transient increase in the C/EBPβ and C/EBPδ expression. C/EBPβ and C/EBPδ induce low levels of PPARγ and C/EBPα, which can then induce each other's expression in a positive feedback loop that promotes and maintains the differentiated state for the life of the adipocyte.[46]

The Krüppel-like factors (KLFs) are proadipogenic C2H2 zinc-finger proteins that regulate proliferation and differentiation. KLF5 expression is induced early during 3T3-L1 adipocyte differentiation by C/EBPβ and C/EBPδ. Oishi et al. demonstrated that KLF5 could directly upregulate the expression of PPARγ2 and C/EBPα, promoting adipocyte differentiation even in the absence of hormonal stimulation,

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Table 2. Potential Carrot microRNAs to Modulate REACTOME Signaling Pathways Involved in Metabolic Syndrome[a]

REACTOME pathway	predicted human target genes	carrot microRNA
R-HSA-400206—regulation of lipid metabolism by PPAR-alpha	*SLC27A1, CHD9, PEX11A, RORA, AHR, NRF1, NPAS2, HELZ2, RXRB, MED11, GLIPR1, SIN3A, CD36, PPARGC1B, FDFT1, NCOA1, MED1, CDK19, NFYB, TXNRD1, NCOA3, NR1H2, CYP4A11, MED9, MED29, MED13L, MED28, NCOR2, MED23, MED22, TBL1XR1, ACOX1, MED21, MTF1, AHRR, RGL1, PPARA, CLOCK*	gma-miR1511, carrot-m0006-5p, carrot-m0005-3p, carrot-m0016-5p, sly miR166c-5p, bra-miR408-5p, ppt-miR319d-3p, sly miR395b, stu-miR156d-3p, aly miR165a-3p
R-HSA-9006936—signaling by TGFB family members	*ACVRL1, CCNT2, NEDD4L, LTBP2, FURIN, F11R, CHRDL1, ACVR1C, PMEPA1, ITGAV, JUNB, SMAD2, WWTR1, SMAD1, TGFB2, SMAD4, TGIF2, SMAD3, PARP1, TGFB3, STAT1, NOG, SMAD9, INHBA, ACVR2B, TGFBR1, ACVR2A, UCHL5, TGFBR2, CDK9, NCOR2, ZFYVE16, COL1A2, TFDP2, TRIM33, UBE2M, BMPR1A*	aly miR319b-5p, carrot-m0025-3p, sly miR395b, gra-miR7494c, nta-miR479a, osa-miR156l-3p, ata-miR166b-5p, zma-miR164g-3p, ppt-miR390b
R-HSA-1489509—DAG and IP3 signaling	*AHCYL1, PDE1B, PRKCE, PDE1A, PRKCD, CAMK2A, ITPR1, PRKX, ADCY1, ADCY7, ADCY6, CAMKK2, CREB1, ADCY9, PRKAR2A, CAMK4, PLCG1*	carrot-m0032-5p, osa-miR156l-3p, mtr-miR168a, ppe-miR396a, mdm-miR396a, aly miR165a-3p
R-HSA-163685—integration of energy metabolism	*AHCYL1, PRKAA2, SLC2A1, ITPR1, CACNA1A, CACNA1C, ADCY1, IQGAP1, ADIPOR1, CACNA1E, ADCY7, ADCY6, GNAI2, GNG2, PPP2R1B, PPP2R1A, GNG4, PRKAR2A, GNG7, MLX, CD36, PKLR, CACNA2D2, ACSL4, ADRA2C, MLXIPL, CACNB3, PLCB3, ADCY9, GNAS, GNB5, PLCB1, STX1A*	carrot-m0031-3p, aly miR165a-3p, cca-miR396a-3p, carrot-m0010-3p, peu-miR2912a, carrot-m0053-5p, gra-miR7494c, carrot-m0025-3p, osa-miR156l-3p
R-HSA-381340—transcriptional regulation of white adipocyte differentiation	*CEBPA, CEBPD, CHD9, HELZ2, MED11, CD36, PCK1, NCOA1, MED1, CDK19, NCOA3, EBF1, MED9, NR2F2, MED29, NFKB1, MED13L, MED28, NCOR2, MED23, MED22, KLF5, TBL1XR1, MED21, PLIN1, PPARA*	ptc-miR3991, gma-miR156n, carrot-m0005-3p, pta-miR396, carrot-m0032-5p, ata-miR172c-5p, ppt-miR477e, zma-miR164g-3p, carrot-m0009-3p

[a]For more detailed information about the predicted target gene and microRNA, refer Table S2.

while downregulation inhibited the adipocyte differentiation.[47] The KLF6 inhibits the expression of the proto-oncogene Delta-like 1/preadipocyte factor-1 (DLK1/PREF1), a gene encoding a transmembrane protein that inhibits adipocyte differentiation.[48] Li et al. reported that following the differentiation by hormonal stimulation, the 3T3 fibroblast line with a forced tetracycline-regulated KLF6 expression markedly reduced the level of DLK1 expression. Although forced expression of KLF6 is insufficient to promote adipocyte differentiation, cells with reduced amounts of KLF6 show decreased adipogenesis.[48] Interestingly, in adipose-derived stem cells, the overexpression of KLF6 increased the number of lipid droplets and the expression of PPARγ and C/EBPα. In contrast, the inhibition of KLF6 significantly decreased the accumulation of lipid droplets and inhibited PPARγ and C/EBPα expression in stem cells. These results indicated that KLF6 is a positive regulator of adipogenesis and lipid accumulation.[49]

CD36 is a class B scavenger receptor implicated in the pathogenesis of metabolic disorders such as obesity, insulin resistance, and atherosclerosis because it binds long-chain fatty acids and facilitates their transport into cells, thus participating in muscle lipid utilization, adipose energy storage, and gut fat absorption.[50] Christiaens et al. evaluated the effect of CD36 on the adipogenesis process in an in vitro model and *CD36* knockdown mice with diet-induced obesity. The authors reported that during the differentiation of 3T3-F442A preadipocytes, silencing of CD36 resulted in impaired cell differentiation, with a significantly lower intracytoplasmic lipid content, a higher PREF-1 expression, and a lower expression of aP2 and PPARγ. The animal model presented a significantly lower fat mass than the wild type, indicating impaired adipogenesis and fat pad formation. Also, total and HDL cholesterol levels were increased in the plasma of the CD36 knockdown mice.[50]

These results were corroborated by Gao et al. in a 3T3-L1 cell culture. After the adipogenic induction, the CD36 protein level and PPARγ, C/EBPα, and FABP4 levels were increased. The suppression of *CD36* decreased the number of mature adipocytes and, thus, the lipid accumulation. The *CD36* knockdown also significantly suppressed the expression of the adipogenic markers *FABP4*, *C/EBPα*, *PPARγ*, and mitochondrial biogenesis genes such as peroxisome proliferator-activated receptor (PPAR) coactivator-1α (*PGC-1α*) and mitochondrial transcription factor A (*mtTFA*).[51] In addition, Kennedy et al. demonstrated that adipose tissue from *CD36* knockout mice was more insulin-sensitive and had lower levels of inflammatory markers (i.e., IFN-γ, MCP-1) as compared to wild-type mice.[52]

EBF1 is a member of the EBF (O/E) family of helix−loop−helix transcription factors that play a significant role in cellular differentiation and function, including adipogenesis. Indeed, EBF1 appears to induce adipocyte differentiation with similar timing and efficiency as PPARγ2.[53] Åkerblad et al. found that EBF-1 and PPARγ2 induce adipocyte differentiation with comparable kinetics and efficiency. Although distinct gene expression profiles in NIH-3T3 cells were reported, a similar gene expression pattern was found on day 10. However, the authors suggest that these discrete gene expression differences might drive differences in the nature and function of these NIH-3T3-derived adipocytes.[54] Studies using short-hairpin RNA-mediated knockdown indicated that C/EBPα and PPARγ are direct EBF1 targets and that EBF1 likely amplifies the actions of C/EBPβ and C/EBPδ by acting immediately

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Figure 5. Principal diseases associated with the predicted carrot microRNA gene target. (A) Annotation using DAVID: All terms were considered significant, considering at least eight hits and p-value < 0.05. (B) Predicted carrot microRNA target genes filtrated by The Nutritional and Metabolic Diseases class with DisGeNET Cytoscape App v7.3.0.

downstream of those factors, and reduction of EBF1 blocks the 3T3- L1 differentiation. C/EBPβ and C/EBPδ promote the induction of EBF1. These results indicate that EBF1 is required for adipogenesis.[55] Taniguchi et al. reported that *EBF1* inhibition by microRNA-33b decreases lipid accumulation (TGA deposition and oil red O staining) after differentiation of porcine subcutaneous preadipocytes. Consistent with the previous results, the inhibition of EBF1 also downregulated adipogenic genes, such as *PPARγ* and *C/EBPα*. Consequently, PPARγ downstream genes, including *aP2*, *CD36*, and *ADIPOQ*, were downregulated.[56]

Adipogenesis is a complex process that involves diverse transcription factors and several mechanisms. As a key process in determining the number and maturation of adipocytes, it represents a possible therapeutic approach for obesity and metabolic syndrome complications.[57] Interestingly, the early adipogenesis program can be modulated by carrot microRNAs. Carrot microRNAs target factors necessary for cellular determination such as BMPs/SMAD signaling members, C/EBPs, and KLFs. Therefore, it is possible to alter this early programming and modify fat accumulation, fatty transport, sensitivity to insulin, and chronic inflammation, among others.

3.4. Gene−Disease Association. According to the performed annotation of the target genes in the GAD disease class database using DAVID (Figure 5A), the predicted genes (2796 annotated) are mainly involved in metabolic and cardiovascular diseases, and some others related to chemical dependency, representing 31.69, 26.45, and 25.56%, respectively.

Filtrating the mapping DisGeNet results, specifically the class of Nutritional and Metabolic diseases (Figure 5B), there are found diseases such as obesity, diabetes, and insulin resistance including genes such as *KCNMA1*, *CEBPA*, *HTR2A*, *CYP26B1*, *ESR1*, *GNAS*, *AHR*, *KSR2*, *CYCS*, *PLIN1*, *NTRK2*, *HTR2C*, *UCP2*, *PPARA*, *FOXO3*, *IDO1*, *PHF6*, *CASP1*, *FGF21*, and *ZNF169*. These results concord with the functional annotation of the targets and the mapping using PANTHER, DAVID, KEGG pathways, and REACTOME and indicated that the carrot microRNAs can potentially modulate diseases related to metabolic syndrome.

4. PERSPECTIVES

The data reveal the potential applications of carrot microRNAs as effective therapeutics for obesity and associated metabolic diseases. Although the in-silico assays are predictions based on computational analysis using microRNA target prediction algorithms focusing on characteristics of the mRNA sequence and the microRNA-mRNA interaction, it is crucial to acknowledge the inherent challenges in translating these findings into practical applications. Specifically, the cross-

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kingdom regulation of human gene expression by plant-derived microRNAs presents a novel but controversial area of research with significant skepticism surrounding the absorption, stability, and functional impact of these microRNAs in human physiology. This type of analysis allows the screening of a large amount of data and the selection of some candidates with great potential to modulate the biological process of interest.

Predicted mRNA targets and microRNAs must be experimentally verified to obtain complete legitimacy. The need for experimental validation extends beyond confirming bioinformatics predictions; it is essential for establishing the mechanistic basis of how these microRNAs exert their effects across species barriers and assessing their therapeutic potential. Rigorous in vitro and in vivo studies, including the detection of these microRNAs and their target genes in human tissues postcarrot intake, are imperative to substantiate the bioavailability and regulatory capabilities of carrot microRNAs in human metabolic pathways. In the case of the plant microRNAs and their cross-kingdom functionality, some factors that must be clarified involve the validated stability of the microRNA after dietary uptake, evaluation of the direct microRNA-mRNA interaction by direct assays (such as luciferase assays) or indirect assays that evaluated the expression of mRNA or protein using high-throughput technologies (e.g., mass spectrometry).[58] Gain-and-loss-of-function experiments must demonstrate how the microRNAs regulate target protein expression, and the predicted changes in protein expression must be associated with a modified biological function.[59]

Moreover, multidimensional observation of highly microRNA-enriched foods or supplements should be arranged for the effects of microRNAs on animals and patients with different health levels to assess potential risks due to a single microRNA can target multiple target genes, as a single microRNA can target multiple genes, potentially leading to unintended consequences.[60] Future studies will provide definitive evidence of the revolutionary concept of cross-kingdom regulation and its potential for the prevention and treatment of many human diseases.

5. CONCLUSIONS

This study found that carrot microRNAs could modulate critical aspects of signaling pathways involved in the development of different metabolic syndrome factors, such as obesity, NAFLD, insulin resistance, and type 2 diabetes. The emergence of these microRNAs as regulators of metabolism generates great expectations from a clinical and food technology perspective; since they come from foods. Preventive and therapeutic strategies can be developed based on foods enriched with these compounds to manage obesity and metabolic syndrome. Acknowledging the diversity within *Daucus carota* L., future explorations will aim to broaden the spectrum of microRNA sequences analyzed to encompass a wider genetic diversity, thereby enhancing the representativeness and applicability of our findings. Experimental validation, including in vitro and in vivo studies, will be essential to verify the computational predictions regarding the trans-kingdom regulation of carrot microRNAs on human metabolic pathways. Further studies are necessary to examine the absorption, functionality, efficacy, and safety of these novel therapeutic approaches.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09633.

In silico identification of carrot microRNA targets with The psRNATarget Web server and carrot microRNA and their potential human target gene (PDF)

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Notes

The authors declare no competing financial interest.

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