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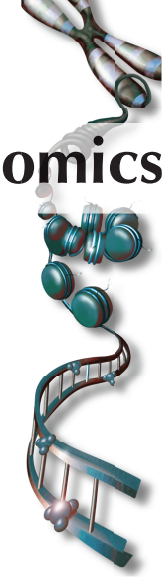

Expression study of candidate miRNAs and evaluation of their potential use as biomarkers of diabetic neuropathy

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Aim: To evaluate the expression of candidate miRNAs in relation to diabetic polyneuropathy (DPN) and cardiovascular autonomic neuropathy (CAN). **Materials & methods:** The expression of six candidate miRNAs has been evaluated in 49 Type 2 diabetes patients with neurological evaluation. **Results:** A higher expression of miR-128a was seen in patients with DPN compared with those without DPN ($p = 0.015$). miR-155 and miR-499a seemed to be down-expressed in patients with DPN ($p = 0.04$ and $p = 0.05$, respectively). A lower expression of miR-155 ($p = 0.05$) was observed even in patients with CAN with respect to CAN-negative. A higher expression of miR-155 was associated with the rs767649 polymorphism variant allele compared with the wild-type allele ($p = 0.03$). **Conclusion:** miR-128a, miR-155 and miR-499a might be involved in diabetic neuropathies development.

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Keywords: diabetic neuropathy • expression study • microRNAs

Diabetic neuropathy (DN) is a common complication of diabetes, which affects up to 50% of patients with diabetes [1]. Among the heterogeneous DNs, diabetic distal symmetric sensorimotor polyneuropathy (DPN) and diabetic cardiovascular autonomic neuropathy (CAN) are the most frequent forms, with a prevalence of 30 and 20%, respectively, and both can exert a heavy impact on quality of life, morbidity and mortality [1,2]. The etiopathogenesis of DPN and CAN have not yet been fully understood, but there is a common view that these complications are multifactorial diseases, triggered by the interaction between predisposing genetic, epigenetic and environmental factors. Although hyperglycemia, duration of diabetes, hypertension, dyslipidemia and obesity are recognized as the principal causes of the development of DN, clinical trials have shown that the development of these complications cannot be completely prevented by the control of risk factors, suggesting that genetic factors can play a key role [3]. Indeed, many recent studies have described genetic variations associated with the development of these complications, but only very few genes have been extensively investigated in different populations and confirmed in large cohorts [4].

miRNAs are small (18–22 nucleotides) noncoding RNA molecules, that regulate gene expression at the post-transcriptional level [5] and their alterations may also be involved in the development of various diseases such as cancer, heart, metabolic and inflammatory diseases. In particular, there is increasing evidence of miRNA involvement in development of diabetes and its related complications, including DN [6].

Recently, the association of *MIR128A*, *MIR146A*, *MIR27A* and *MIR499A* polymorphisms with the risk of developing DNs in an Italian cohort of patients with Type 2 diabetes (T2D) has been described [7,8]. The T allele of rs11888095 single nucleotide polymorphism (SNP) in *MIR128A* was significantly more frequent in T2D patients with DPN and correlated with a higher DPN severity. The variant allele of rs2910164 SNP in *MIR146A* seemed to have a protective role for both DPN and CAN. Conversely, the variant allele of rs895819 SNP in *MIR27A* resulted

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as a risk allele for the development of early CAN [7]. Moreover, the variant allele of rs3746444 in the *MIR499A* increases the susceptibility to both forms of neuropathy. In particular, carriers of the GG genotype had a greater risk of developing both DPN and CAN and the correlation with several neurological parameters seemed to indicate a dose-dependent effect of the G allele, related to the severity of neuropathy [8].

Many studies have instead concentrated on the analysis of miRNA expression profiles in different tissues implicated in diabetic disease, such as the pancreas, the adipose tissue and the liver. In diabetic patients, numerous miRNAs have been observed with altered expression: miR-375, involved in insulin secretion [9], miR-27a, which shows its effects on adipose tissue [10], miR-21, involved in β-cell differentiation of pancreatic progenitor cells [11], as well as many other miRNAs. Investigations on miRNA molecules have been also conducted in DN to evaluate their possible role as biomarkers or therapeutic targets [12]. For example, mir-146a seems to modulate inflammatory response in DPN and it is downregulated in the sciatic nerves of mice with DPN compared with a healthy control group [13–15].

Despite these first results, the studies conducted to analyze possible miRNA expression variations in relation to the development of DN are still poor. Here, our aim was to investigate the expression level of six candidate circulating miRNAs in a cohort of 49 Italian patients with T2D, evaluated for CAN and DPN, and in 15 healthy subjects. We selected mir-128a, mir-27a, mir-146a and mir-499a as candidate miRNAs because of previously observed associations of some polymorphisms in these miRNAs genes with DN [7,8]. Mir-21 and mir-155 were selected because these two miRNAs seem to be altered in painful peripheral neuropathies [16]. We also aimed to determine if any observed deregulation was associated to different forms of DN (CAN or DPN) or to both of them. In addition, we studied the correlation of miRNAs expression with common polymorphisms localized on these miRNA genes, to explore a possible functional role of their genetic variants.

Materials & methods

Patients recruitment

A total of 49 patients were consecutively recruited among the patients attending the diabetic clinic of the Tor Vergata University Hospital in Rome (Italy) who underwent neurological evaluation. The inclusion criteria were the diagnosis of T2D, according to American Diabetes Association diagnostic criteria [17] and age between 18 and 80 years. The patients with peripheral or autonomic neuropathies from other causes than diabetes and with other conditions potentially responsible for autonomic dysfunction were excluded from recruitment. Other exclusion criteria have been reported in detail in our previous study [8]. A total of 15 healthy individuals without a diagnosis of diabetes were also enrolled as control subjects. They were anonymized blood donors; thus, clinical information is unavailable. The study was approved by the ethics committee of the Tor Vergata University Hospital. All participants provided written informed consent.

Clinical & neurological evaluation

A complete clinical history was recorded regarding diabetes, comorbidities, cardiovascular disease and any potential cause of neuropathy. During the neurological evaluation, height, weight, waist circumference, casual blood pressure and blood glucose were also measured for each patient. Routine laboratory and neurological assessments performed on all patients were described in a previous paper [7].

Briefly, neurological examination included assessment of neuropathic symptoms and deficits (using the Michigan Neuropathy Screening Instrument Questionnaire [MNSI-Q] and the Michigan Diabetic Neuropathy Score [MDNS] [18]), vibration perception threshold, and cold and warm thermal perception thresholds. According to Toronto Consensus on DN, criterion for the definition of DPN (probable) was the presence of at least two abnormalities among neuropathic symptoms or signs which are vibration perception threshold and thermal perception thresholds [19].

The definition of CAN (early) was determined by the presence of at least one abnormal cardiovagal test among four cardiovascular autonomic tests (heart rate response to deep breathing, lying to standing and to Valsalva maneuver and the orthostatic hypotension test) performed in accordance with standard procedure and using age-related reference values [2,19].

mRNA isolation & measurement of miRNA expression

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) using TRIzol reagent (Invitrogen-ThermoFisher, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were

evaluated by using a NanoDrop ND-1000 Spectrophotometer (EuroClone SpA, Milan, Italy). RNA integrity was confirmed by gel electrophoreses. For mature miRNA expression analysis, cDNA was synthesized using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems-Thermofisher, CA, USA). Quantitative real-time PCR analysis was performed using the 7500 Real-Time PCR System (Applied Biosystems). MiR-499a-5p, miR-27a-5p, miR-146a-5p, miR-128a-5p, miR-21-5p and miR-155-5p expression analysis was performed using Taqman Advanced miRNA Assay (Applied Biosystems). For normalization, let-7d-5p assay was used as endogenous control. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [20].

Genotyping analysis

Genomic DNA was isolated from peripheral blood with the Qiagen Blood DNA Mini Kit (Qiagen, Hilden, Germany), according to the standard procedure. Genotyping of selected genetic variant in miRNA genes was performed by allelic discrimination assay by TaqMan technology using a minor groove binder-specific allelic probe and 7500 real-time instrument (Applied Biosystems) for rs3746444 (*MIR499A*), rs895819 (*MIR27A*), rs2910164 (*MIR146A*), rs1188809 (*MIR128A*) and rs767659 (*MIR155*). Direct sequencing was performed for rs1292037 (*MIR21*).

Statistical analysis

The miRNAs expression levels of each sample were analyzed in triplicate and data were reported as mean values ± standard deviation. The ANOVA test was used to compare expression data among the different phenotypic and genotypic groups. A p-value ≤ 0.05 was considered as significant. The receiver operating characteristic (ROC) curve was performed to evaluate the ability to discriminate the neuropathic group using miRNAs that were significantly altered. All statistical analyses were performed by the SPSS program, version 19 (IBM Corp, NY, USA).

Results

Clinical characteristics of participants

We included 49 participants with T2D (32 men), with a mean age of 62.5 ± 6.6 years, a diabetes duration of 12.6 ± 9.2 years, a BMI of 31.6 ± 6.2 kg/m2, and HbA1c of 7.2 ± 1.5% (54.88 ± 16.38 mmol/mol). All 49 diabetic patients included in the study underwent neurological assessment, among which 28 (57%) satisfied the diagnostic criteria for DPN and 23 (47%) the diagnostic criteria for CAN (Table 1). In 19 patients, there was coexistence of DPN and CAN, while 17 patients were free from both DPN and CAN. Patients with DPN had longer diabetes duration (15.1 ± 10.4 vs. 9.3 ± 5.8 years, p = 0.026), were less commonly alcohol consumers (p = 0.008), were more often under insulin treatment (p = 0.0001) and with peripheral arterial disease (p = 0.04) and diabetic retinopathy (p = 0.003) compared with those without DPN (Supplementary Table 1). Similarly, presence of CAN was associated with insulin treatment (p = 0.04) and diabetic retinopathy (p = 0.022), with a lower percentage of alcohol consumers (p = 0.003) and of physically active participants (p = 0.024), and with lower total cholesterol levels (p = 0.006) (Supplementary Table 2).

Expression levels of miRNAs in neuropathic subgroups of T2D patients & in healthy controls

The six candidate miRNAs expression levels were analyzed in 49 T2D patients. Statistical analysis has been performed by splitting diabetic patients into different groups based on the presence or absence of DPN, CAN or at least one form of DN (complete results are shown in Supplementary Table 3).

As shown in Figure 1, in DPN patients the mean level of miR-128a expression was higher compared with the group of patients without DPN (p = 0.015). In contrast, for miR-155 and miR-499a, a lower expression was observed in patients with DPN compared with the group of patients without DPN (p = 0.04 and p = 0.05, respectively). The differences of expression of miR-128a and miR-155 were statistically significant also after correction for sex, age and diabetes duration (P_{adj} = 0.04 and P_{adj} = 0.05, respectively). Moreover, the expression of miR-155 was lower also in patients with CAN with respect to those without CAN with a borderline significance after adjustment for sex, age and diabetes duration (p = 0.05 and P_{adj} = 0.06). Comparing diabetic patients with and without DN, we observed a significant difference in the expression of miR-128a (p = 0.05), miR-155 (p = 0.014) and miR-499a (p = 0.045). The difference of miR-155 expression continues to be significant after correction for sex, age and diabetes duration (P_{adj} = 0.017). For the other miRNAs, no significant differences in expression between the different groups were observed (Supplementary Table 3).

Table 1. Clinical and anthropometric characteristics of the 49 patients with Type 2 diabetes examined in this study.

Females/males	17/32
Age (years)	62.5 ± 6.6
Disease duration (years)	12.6 ± 9.2
BMI (kg/m^2)	31.6 ± 6.2
Insulin treated (%)	20.4
HbA1c (%)	7.2 ± 1.5
HbA1c (mmol/mol)	54.88 ± 16.38
Total cholesterol (mg/dl)	170.7 ± 37.7
HDL cholesterol (mg/dl)	46.7 ± 13.9
Triglycerides (mg/dl)	147.1 ± 133.6
Serum creatinine (mg/dl)	0.96 ± 0.2
With microalbuminuria (%)	27.1
Casual systolic blood pressure (mmHg)	138.6 ± 15.3
Casual diastolic blood pressure (mmHg)	81.5 ± 17.1
With hypertension (%)	81.6
With peripheral arterial disease (%)	10.2
With diabetic retinopathy (%)	26.7
With cardiovascular disease (%)	16.3
Current smokers (%)	58.3
Regular physical activity (%)	66.7
Alcohol consumption (%)	38.1

HDL: High-density lipoproteins.

To assess whether the observed dysregulation of miRNAs is effectively linked to the presence of neurological complications and not to the diabetic state, we have then analyzed these miRNAs in 15 healthy subjects and compared the expression levels with the whole group of patients with T2D. There were no significant differences between T2D patients and healthy controls (Supplementary Table 4). On the contrary, significant differences for miR-128a and miR-155 have been observed in subgroups of patients with DN compared with healthy controls (Figure 2). MiR-128 showed significant differences in expression between the healthy control group and both neuropathic groups ($p = 0.038$ and $p = 0.05$, respectively for DPN and CAN), but there were no significant differences between healthy subjects and diabetic patients without neuropathy (Figure 2). We also observed that miR-155 expression decreased in both groups with DPN and CAN ($p = 0.018$ and $p = 0.012$, respectively for DPN and CAN), and in the DN group ($p = 0.015$) compared with healthy subjects. On the contrary, this reduction was not observed in patients without neuropathic complications (Figure 2).

Relation between miRNA expression & genotypic classes

To verify the possible association between common polymorphisms localized in the miRNA genes and the different expression levels of the same miRNAs, we compared the distribution of the mean values of miRNA expression in the different genotypic classes for each SNP analyzed, in the whole cohort of patients with T2D, who underwent neurological evaluation. As shown in Table 2, the rs767649 polymorphism variant allele in the miR-155 promoter region was associated with a higher expression of this miRNA ($p = 0.003$) compared with the wild-type allele, also after correction for sex, age and diabetes duration ($P_{adj} = 0.013$). No significant association was observed between the rs11888095 polymorphism in the miR-128a and the expression levels of the respective miRNA ($p = 0.29$), but it was possible to observe a linear increase in miR-128a expression levels among the three genotypic groups. This linearity reached statistical significance after correction for sex, age and diabetes duration ($P_{adj} = 0.022$).

Receiver operating characteristic curve analysis

For the miRNAs that showed significant differences in expression levels in patients with T2D according to the presence of DPN and CAN, we performed the receiver operating characteristic (ROC) curve analysis, to evaluate the capacity of these miRNAs to identify the patients with T2D more likely to develop neuropathy. We tested a first predictive model for DN, combining the expression levels of miR-128a, miR-155 and miR-499a, and a second

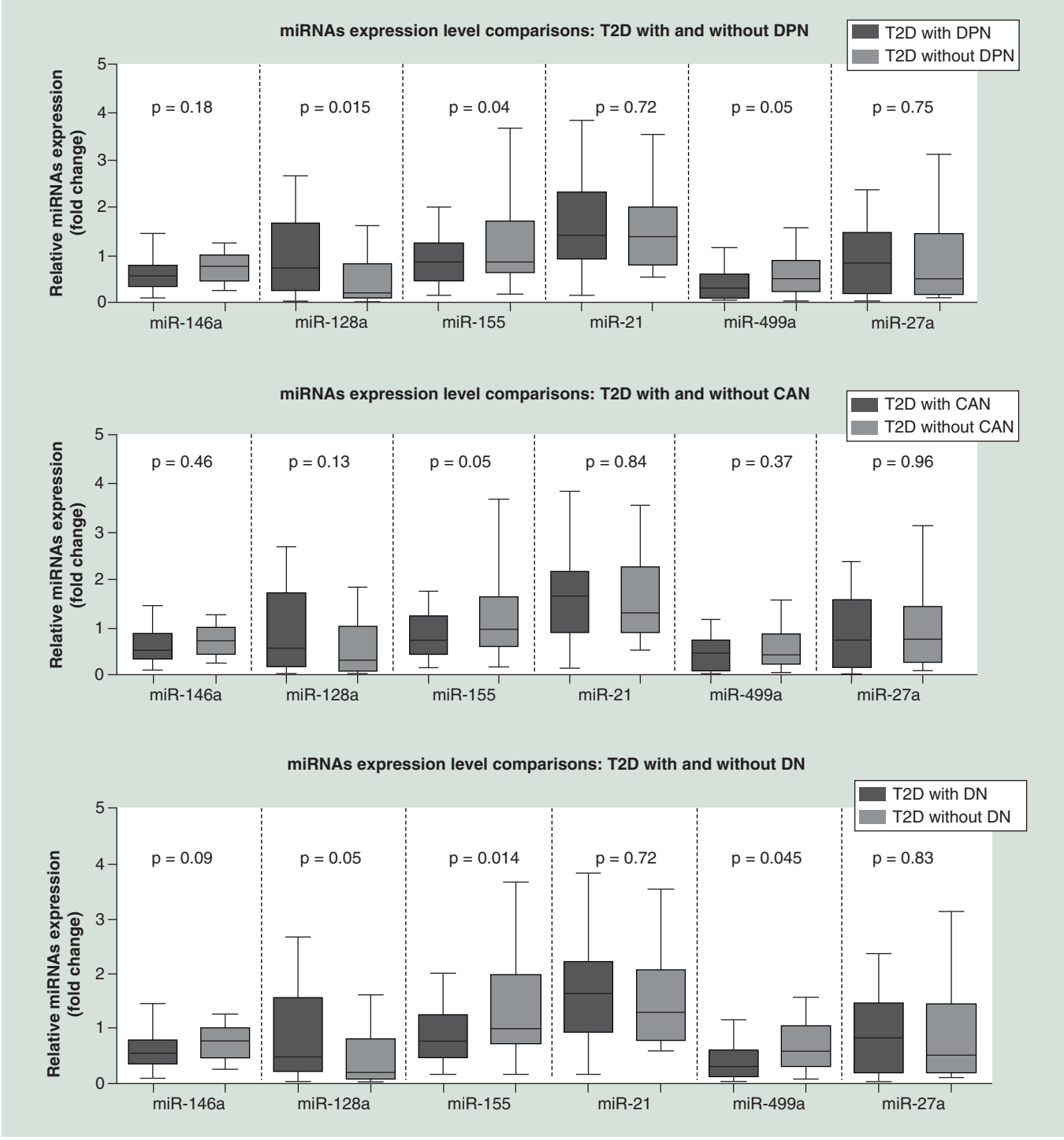

Figure 1. Distribution of miRNAs expression levels in different subgroups of diabetic patients.
CAN: Cardiovascular autonomic neuropathy; DN: Diabetic neuropathy; DPN: Diabetic polyneuropathy; T2D: Type 2 diabetes.

predictive model including only the two miRNAs with more significant differences (miR-128a and miR-155). The results demonstrated that the area under the ROC curve (AUC) for the first model was 0.817 with 75.9% sensitivity and 76.5% specificity, while the AUC for the second model was 0.802 with 80.6% sensitivity and 70.6% specificity (Figure 3). Subsequently, we evaluated the ability of these models to predict DPN in diabetic patients. In the first model, combining the expression levels of miR-128a, miR-155 and miR-499a, the AUC was 0.802 with

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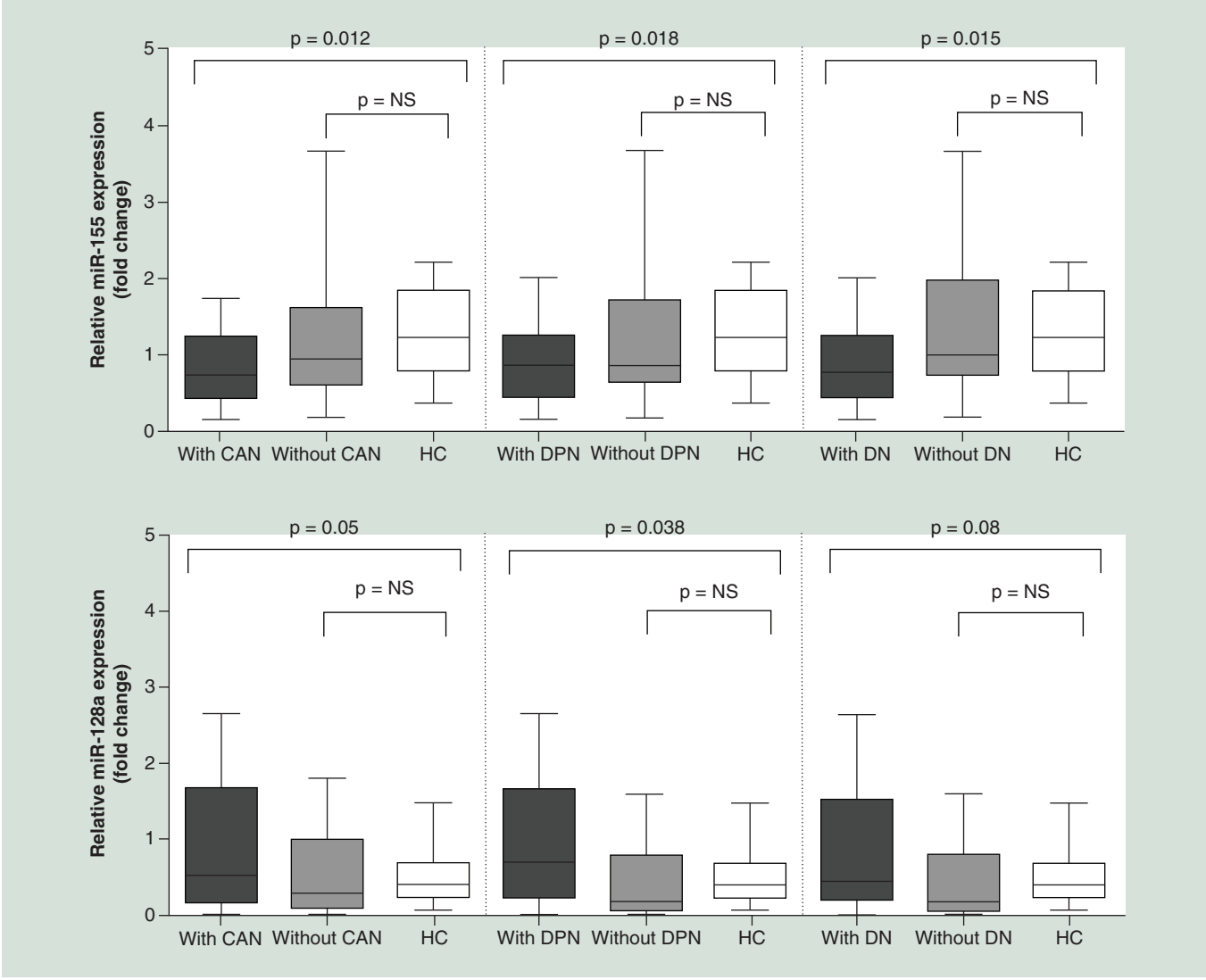


Figure 2. Comparisons of miR-155 and miR-128a expression levels between subgroups of diabetic patients (DPN, CAN, DN) and HC.
CAN: Cardiovascular autonomic neuropathy; DN: Diabetic neuropathy; DPN: Diabetic polyneuropathy; HC: Healthy controls; NS: Not significant.

74.1% sensitivity and 76.2% specificity, while the AUC for the second model was 0.802 with 80.6% sensitivity and 70.6% specificity (Figure 3). Also for the DPN, the two models examined have a similar discriminating power. The same models have been tested to discriminate patients with and without CAN, but neither of them turned out to be predictive; this finding was quite expected considering that only miR-155 had shown a borderline association with CAN.

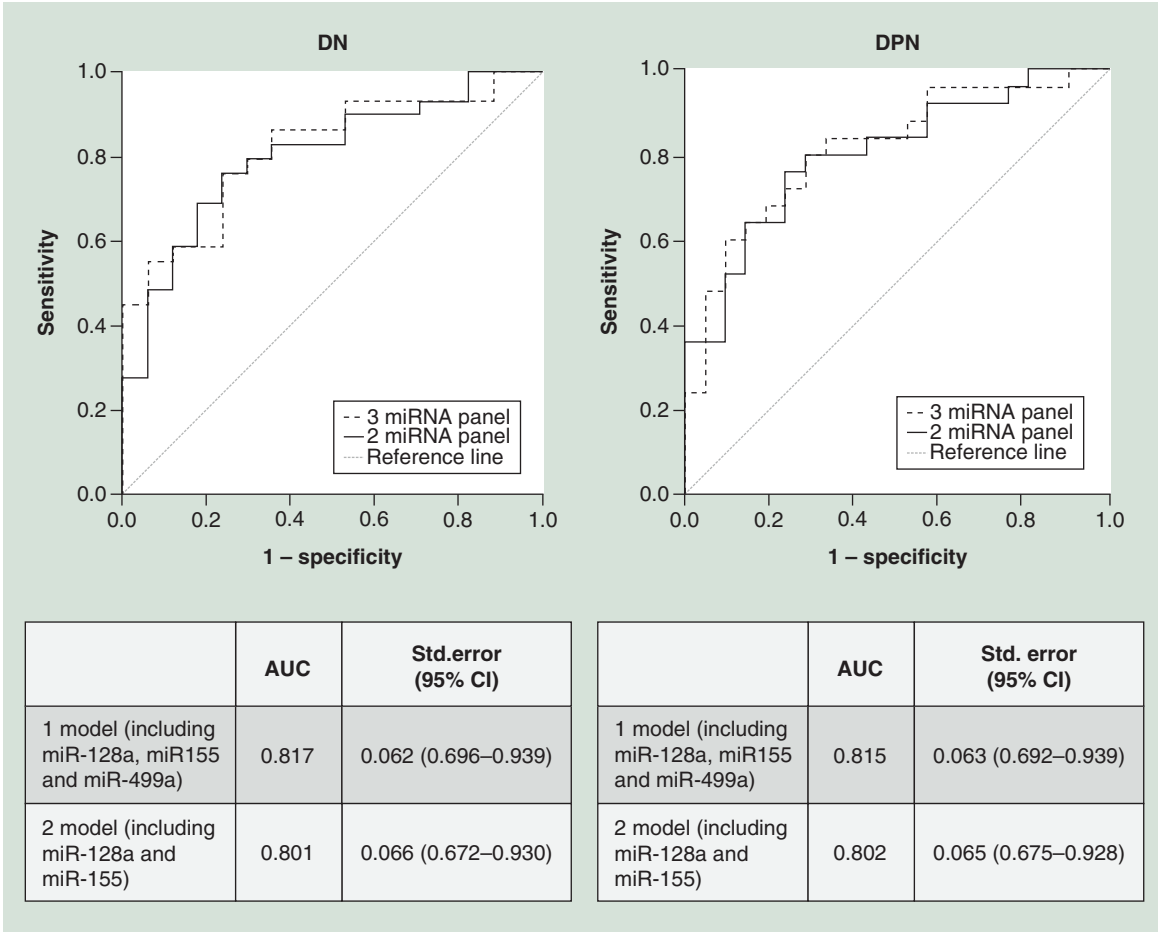
Discussion

DN is a common microvascular complication of diabetes with complex, multifactorial pathogenetic mechanisms, which have to be further studied to be fully understood. DPN and CAN are the most common forms of DN and the identification of potential genomic biomarkers could be useful to discriminate patients more susceptible to develop these severe neuropathic forms. In last years, many studies have been conducted to establish whether miRNAs could be used as biomarkers for the diagnosis, prognosis and treatment of various diseases, including diabetes and its complications [12]. We investigated the expression of six candidate miRNAs (miR-21, miR-27a, miR-128a, miR-155, miR-146a and miR-499a) in 49 T2D patients well characterized for DPN and CAN.

Table 2. miRNA expression levels in different classes of genotypes for the investigated SNPs in patients with Type 2 diabetes.

		Genotypic classes		p-value	P_{adj} †
rs1292037	TT	TC	CC		
miR-21	1.58 ± 0.91	1.61 ± 0.79	1.19 ± 0.84	0.81	0.41
rs895819	TT	TC	CC		
miR-27a	0.97 ± 0.84	0.71 ± 0.46	-	0.26	0.46
rs11888095	CC	CT	TT		
miR-128a	0.67 ± 0.69	0.73 ± 0.73	1.28 ± 1.08	0.29	**0.022**
rs2910164	CC	CG	GG		
miR-146a	0.68 ± 0.34	0.69 ± 0.33	0.55 ± 0.32	0.72	0.68
rs767649	TT	TA	AA		
miR-155	0.98 ± 0.67	2.14 ± 1.06	-	**0.003**	**0.013**
rs3746444	AA	AG	GG		
miR-499a	0.44 ± 0.40	0.58 ± 0.39	0.47 ± 0.35	0.47	0.61

Expression levels are reported as mean ± SD.
Significant differences are reported in bold.
† p-values after correction for age, sex and diabetic disease duration.
SNP: Single nucleotide polymorphism.



	AUC	Std.error (95% CI)
1 model (including miR-128a, miR155 and miR-499a)	0.817	0.062 (0.696–0.939)
2 model (including miR-128a and miR-155)	0.801	0.066 (0.672–0.930)

	AUC	Std. error (95% CI)
1 model (including miR-128a, miR155 and miR-499a)	0.815	0.063 (0.692–0.939)
2 model (including miR-128a and miR-155)	0.802	0.065 (0.675–0.928)

Figure 3. Receiver operator characteristic curves for diabetic neuropathy (with vs without) and diabetic polyneuropathy (with vs without).
AUC: Area under the curve; CI: Confidential interval; DN: Diabetic neuropathy; DPN: Diabetic polyneuropathy; Std: Standard deviation.

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In our study, we observed that expression levels of miR-128a, miR-155 and miR-499a are significantly different in patients with DPN and CAN compared with those without neuropathy.

In particular, miR-128a is significantly upregulated in DPN patients, compared with the group of patients without DPN. This miRNA seems to be involved in carbohydrates metabolism by downregulating genes of insulin signaling pathway like *INSR, IRS1, PIK3r1* [21]. We could hypothesize that the increased miR-128a expression observed in DPN patients might exacerbate insulin resistance typical of T2D and favor in this way the development of DN. Moreover, miR-128a has been found to play a regulatory role in adipogenesis and lipolysis through downregulation of *PPARG* and *SERTAD2* [22–24].

We highlighted an association between rs11888095 SNP in *MIR128A* (previously associated with a higher risk of developing DPN [7]) and the expression level of this miRNA. This finding suggests that the variant allele is associated with a higher risk to develop DPN, because it is involved with the miRNA expression. To our knowledge, our study is the first to show a correlation between rs11888095 SNP and the miR-128a expression.

MiR-155 resulted downregulated in DN patients compared with both T2D patients without neuropathy and healthy controls. The reduction of this miRNA expression was present in both neuropathy forms, CAN and DPN. MiR-155 has been reported to regulate multiple biological pathways simultaneously. Experimental studies have shown that this miRNA is a positive regulator of glucose tolerance and insulin sensitivity *in vivo*, through the downregulation of its targets: *SOCS1*, *C/EBPβ* and *HDAC4* [25]: the loss of the miR-155 determines hyperglycemia, glucose intolerance and insulin resistance. The decrease in miR-155 expression observed in neuropathic patients could therefore be associated with a decrease in glycogenesis and an increase in blood glucose. A downregulation of miR-155 was also observed in another microvascular complication of diabetes, diabetic nephropathy [26]. The miR-155 is involved in regulation not only in the metabolic pathway, but also inflammation, immune system development and function [27]. Chronic low-grade inflammation is now considered one of the critical pathways in the pathogenesis of DN [28] with also a preferential association of inflammatory markers with painful compared with painless DPN [29]. It is possible to hypothesize that miR-155 expression changes could be involved in DN inflammatory and immune mediated pathogenetic mechanisms. Moreover, Harrison *et al.* observed that miR-155 knock-out mice had decreased IFN-α and IFN-β levels in the hippocampus and increased neuronal degeneration [30] and reduced systemic levels of miR-155 in subjects with peripheral neuropathies [16], suggesting a neuroprotective role for miR-155.

We also observed an association between rs767649 SNP in *MIR155* and the expression levels of this miRNA. In particular, subjects with variant allele showed a higher expression of miR-155 compared with wild-type subjects. This SNP is localized on promoter region of *MIR155* gene and was reported to be capable of affecting the binding affinity of NF-κB [31,32]. We could speculate that rs767649 variant allele might enhance the binding of transcriptional factors and cause an increase of miR-155 expression. In a previous study, an association of the rs767649 variant allele with the susceptibility to T2D was observed [33]. However, in the current study the miR-155 expression was just a little higher in healthy controls than in T2D, without reaching a statistical significance. This could be due to the small number of healthy subjects analyzed for miRNAs expression, but we might also suppose that other factors (besides the rs767649 SNP) could modulate the expression of this miR in DN.

Patients with DN showed a lower expression of miR-499a with respect to DPN-negative patients, although with a borderline significance. However, it was not possible to find a statistically significant link between the miR expression and the presence of the rs3746444 polymorphism, whose variant allele seemed to be associated with higher risk of CAN and DPN in a previous study [8]. We can suppose that other mechanisms can contribute to alter the expression of the miR, or that the variant allele could be involved in a change in the specific miRNA targets rather than in expression regulation. Therefore, regarding the contribution of miR-499a in the development of DN we can only speculate. MiR-499a could regulate the apoptotic pathway involving CnA and Drp1. A lower expression of miRNA should be responsible for a greater activation of Drp1 and therefore an increase in mitochondrial fission and apoptosis [34]. Mitochondria play an important role in neuronal mechanisms and their alterations have been observed in most neurodegenerative diseases [35]. Furthermore, it has been observed that mitochondrial fission promotes reactive oxygen species production and oxidative damage in response to neuronal damage, both mechanisms are involved in the pathogenesis of DN.

It is interesting to note that these three miRNAs observed deregulated in patients with diabetic neuropathy, miR-128a, miR-155 and miR-499a, are implicated in a common pathway: the insulin sensitivity [21,25,36]. Insulin resistance is one of the main features of T2D and the subsequent hyperglycemia is closely associated with DN. Based on this, our data support the main role that poor glycemic control play in the onset of DN.

Although in a previous study, we observed associations of a polymorphism in *MIR27A* gene with CAN susceptibility and a polymorphism in *MIR146A* gene with both CAN and DPN susceptibility [7], at expression levels we did not find any association. Regarding miR-146a we observed only a weak association with DN after correction for sex, age and diabetic disease duration ($p = 0.05$, Supplementary Table 3).

In summary, our data suggest the alteration of miR-128a, miR-155 and miR-499a levels in patients with T2D and DN. Despite the expression levels of circulating miRNAs could be influenced by different factors, some of their features (such as tissue-specificity and the accuracy of technologies used for their quantification and their stability in serum) candidate them as potential predictive biomarkers for different diseases, including DN. In this regard, to evaluate the ability of the expression levels of the three miRNAs previously described to discriminate neuropathic subjects from non-neuropathic subjects, we performed the ROC curve analysis. We evaluated the discriminating potential of two model: the model 1 including the set of the three miRNAs and the model 2 including only the two miRNAs that showed strongly significant expression differences between the various groups (miR-128a and miR-155). Since the two examined models have a similar discriminating power, we consider that it might be sufficient to consider only the model 2, because it seems to identify about the same percentage of neuropathic subjects with only two miRNAs.

Even if the limit of our study is represented by the small sample size, it is important to highlight that our cohort of patients is well clinically characterized and homogeneously and accurately diagnosed. The fact that we do not have data about healthy control subjects could represent another limitation since this group is composed of blood donors. Our findings deserve, of course, further confirmations in larger cohorts, in particular in patients displaying only a specific DN form.

Conclusion

In conclusion, in the complex scenario of DNs this study offers support to the role of miR-128a, miR-155 and miR-499a as epigenetic risk biomarkers of neuropathic complications, opening new prospective to research regarding their role in pathogenic mechanisms. This could open the way to studies finalized to verify the possibility to use them as therapeutic targets.

Future perspective

DNs represent one of the most severe complication of diabetes and emerging evidences indicate that miRNAs could contribute to the susceptibility to these pathologies. Our study also suggests a role of candidate miRNAs in DN. These results could provide the inspiration for further studies. In particular, future studies could consider miRNA enrichment analysis, in order to investigate further biological functions of our miRNAs. Furthermore, to better understand the functional role of these miRNAs and the role of their variability in the development of neurological complications, molecular and functional studies are necessary, with particular focus on the miRNA targets. These further studies, accompanied by replication studies in other populations, could contribute to evaluate these miRNAs as biomarkers of DN.

Summary points

- Diabetic polyneuropathy (DPN) and cardiovascular autonomic neuropathy (CAN) are the most common forms of diabetic neuropathy in patients with Type 2 diabetes.
- Alterations in miRNAs were reported in different disorders including diabetes and its complications.
- Our aim was to analyze the expression of candidate miRNAs in patients evaluated for both forms of diabetic neuropathy.
- We extracted RNA from peripheral blood mononuclear cells and quantified the expression of six miRNAs in 49 T2D patients with neurological evaluation and 15 healthy controls.
- In DPN patients, we observed a higher expression of miR-128a ($p = 0.015$), and a lower expression of miR-155 and miR-499 ($p = 0.04$ and $p = 0.05$, respectively).
- A lower expression of miR-155 ($p = 0.05$) was also seen in patients with CAN respect to CAN-negative patients.
- The rs767649 variant allele in the miR-155 promoter region was associated with a higher expression of this miRNA ($p = 0.003$) compared with the wild-type allele.
- Our data support the involvement of miRNAs in the development of neuropathic complications, particularly in CAN and DPN.

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2019-0242

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study was approved by Ethics Committee of the Tor Vergata University Hospital. All participants provided written informed consent.

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