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Association between a *MIR499A* polymorphism and diabetic neuropathy in type 2 diabetes

Cinzia Ciccacci [a], Andrea Latini [a], Carla Greco [b], Cristina Politi [a], Cinzia D'Amato [b], Davide Lauro [b], Giuseppe Novelli [a], Paola Borgiani [a,*], Vincenza Spallone [b]

[a] Department of Biomedicine and Prevention, Genetics Section, University of Rome "Tor Vergata", Italy
[b] Department of Systems Medicine, Endocrinology, University of Rome "Tor Vergata", Italy

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ABSTRACT

Aims: Diabetic polyneuropathy (DPN) and cardiovascular autonomic neuropathy (CAN) affect a large percentage of diabetic people and impact severely on quality of life. As it seems that miRNAs and their variations might play a role in these complications, we investigated whether the rs3746444 SNP in the *MIR499A* gene could be associated with susceptibility to DPN and/or CAN.
Methods: We analyzed 150 participants with type 2 diabetes. DNA was extracted from peripheral blood samples and genotyping was performed by TaqMan genotyping assay. Cardiovascular tests, MNSI-Q and MDNS for neuropathic symptoms and signs, VPT, and thermal thresholds were used for CAN and DPN assessment. We performed a genotype-phenotype correlation analysis.
Results: We observed that the GG genotype was associated with a higher risk of developing CAN ($P = 0.002$ and $OR = 16.08$, $P = 0.0005$ and $OR = 35.02$, for early and confirmed CAN, respectively) and DPN ($P = 0.037$ and $OR = 6.56$), after correction for BMI, sex, age, HbA1c and disease duration. Moreover, the GG genotype was associated with worse values of MDNS ($P = 0.017$), VPT ($P = 0.01$), thermal thresholds ($P = 0.01$), and CAN score ($P < 0.001$). A logistic multivariate analysis confirmed that *MIR499A* GG genotype, disease duration and HbA1c contributed to early CAN ($R^2 = 0.26$), while the same variables and age contributed to DPN ($R^2 = 0.21$). With a multiple linear regression, we observed that GG genotype ($P = 0.001$) and disease duration ($P = 0.035$) were the main variables contributing to the CAN score ($R^2 = 0.35$).
Conclusions: We described for the first time that the *MIR499A* genetic variation could be involved in diabetic neuropathies susceptibility. In particular, patients carrying the rs3746444 GG genotype had a higher risk of CAN development, together with a more severe form of CAN.

1. Introduction

Diabetic distal symmetric sensorimotor polyneuropathy (DPN) and diabetic cardiovascular autonomic neuropathy (CAN) are the most common forms of diabetic neuropathies.[1] DPN and CAN affect about 30% and 20% of patients with diabetes, reaching a prevalence of >50% in people with higher age and a longer diabetes duration.[2,3] Moreover, both DPN and CAN impact severely on patients' quality of life, survival and health costs.[2–5] Despite the burden of these complications, their pathogenesis has not been fully explored: it is considered multifactorial, with the interaction between genetic and environmental factors and with hyperglycemia playing a leading role. Furthermore, clinical trials have shown that the development of these complications in any single patient cannot be completely anticipated by the control of hyperglycaemia or of other risk factors.[6] Therefore, the role of genetic factors is crucial.

Indeed, in recent years many studies have highlighted how genetic variations can influence the development of these complications.[7,8] However, very few genes have been extensively investigated in different populations and in large cohorts, among which the *ACE* and *MHTFR* genes.[9–15] In particular, polymorphisms in these two genes have been described as being associated with a higher risk of developing DPN.[13–15] On the contrary, only a few studies have reported genetic associations with CAN: for example, *GSTT1* significantly increased the risk of CAN in a Slovak population,[16] while *TCF7L2* polymorphisms seemed to increase CAN risk in an Italian population.[17]

Recently, we described some associations of microRNA gene polymorphisms with both CAN and DPN susceptibility.[18] MicroRNAs or miRNAs are a class of small RNA molecules that function as regulators of gene expression at post transcriptional level. MiRNA biogenesis starts in the nucleus and concludes in cytoplasm, giving rise from a precursor

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* Corresponding author at: Department of Biomedicine and Prevention, Genetics Section, University of Rome "Tor Vergata", 00133 Rome, Italy.
E-mail address: borgiani@med.uniroma2.it (P. Borgiani).

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stem loop structure, to two mature miRNAs: the miR-5p in the sense position and miR-3p in the reverse position. A mature miRNA can regulate the expression of several genes through two main mechanisms: the direct degradation of the target mRNA or by interfering with protein translation.[19]

Since their discovery, it has been evident that miRNAs are implicated in the regulation of a plethora of pathways, such as cellular proliferation and differentiation, signal transduction, inflammation and autoimmunity.[20–22] Many studies have investigated miRNA expression profiles in different tissues involved in diabetic pathology, such as the pancreas, adipose tissue, and the liver.[23–25] Several miRNAs have been reported as dysregulated in diabetic patients, such as miR-375, the first miRNA to be identified as a regulator of insulin secretion,[26] miR-1/133a,[27] miR-29,[28] miR-130,[29] and miR-27,[30] all of which are involved in insulin resistance, as well as many other miRNAs.

Although the majority of studies have focused on miRNA expression profiles, new evidence has pointed out that also polymorphisms in their genes could be of interest. In fact, genetic variations in miRNA genes could alter the maturation of miRNAs themselves and the recognition of their targets[31] and therefore, they could also be involved in disease development. Genetic variations in miRNA genes have been found to be associated with several diseases, such as cancer,[32] cardiovascular diseases[33,34] and autoimmune diseases.[35–38] We have recently described the involvement of *MIR128A*, *MIR146A* and *MIR27A* polymorphisms in the risk to develop diabetic neuropathies in an Italian cohort of patients with type 2 diabetes.[18] The variant allele of rs11888095 SNP in *MIR128A* was significantly associated with a higher risk of DPN (OR = 4.89, P = 0.02) and with a higher DPN severity (P = 0.026). The C allele of rs2910164 SNP in *MIR146A* was associated with a lower risk of developing both DPN (OR = 0.49, P = 0.09) and CAN (OR = 0.32, P = 0.052). On the other hand, the variant allele of rs895819 SNP in *MIR27A* was significantly associated with a higher risk of developing early CAN (OR = 3.43 and P = 0.023).

More recently our interest has shifted towards the miR-499, which is specifically expressed in cardiac cells and skeletal muscle.[39–41] MiR-499 has been reported to play a critical role in both cardiac differentiation[39] and cardiac stress response, preventing cardiomyocyte apoptosis by calcineurin-mediated Drp1 activation and consequent mitochondrial fission.[42] The expression of miR-499 was increased at a circulating level after acute myocardial infarction.[43–45] Recently, it has been described as regulating insulin resistance; indeed, an over-expression of miR-499 is able to enhance the glycogen and improve insulin signaling by PTEN inhibition.[46] Moreover, miR-499 expression levels were increased in hearts and nucleus ambiguus of streptozotocin-induced diabetic rats.[47]

Interestingly, miR-499 is transcribed by two genes - *MIR499A* and *MIR499B* - that are located in the same region in the intron of *MYH7B* gene in chromosome 19, but in the opposite direction. Indeed, *MIR499A* is transcribed in the sense direction, while *MIR499B* is transcribed in the opposite direction. Whereas for miR-499a there are expression data, it is not known if miR-499b is expressed.[48] A common single nucleotide polymorphism (SNP), rs3746444 A > G SNP, is located in the corresponding 3p mature miR-499a region. This SNP has been associated with several diseases, such as cardiovascular diseases,[33] autoimmune diseases[49] and cancer.[32]

Thus, we considered the fact that miR-499 is expressed in the heart and brain (nucleus ambiguus), and is involved in both cardiovascular disease and metabolic syndrome/diabetes as a reason to analyse genetic variability of *MIR499A* with respect to diabetic neuropathic complications. Therefore, our aim was to investigate whether the common polymorphism rs3746444 SNP could be associated with susceptibility to DPN and/or CAN. To this end, we analyzed this SNP in the DNA extracted from peripheral blood samples in a population of 150 participants with type 2 diabetes evaluated for CAN and DPN and subsequently performed a genotype–phenotype correlation analysis.

2. Materials and methods

2.1. Patients recruitment

Patients were consecutively recruited from January 2010 to January 2014 among outpatients attending the diabetic clinic of the Policlinico Tor Vergata in Rome (Italy). The inclusion criteria were a diagnosis of type 2 diabetes and age between 18–80 years. The exclusion criteria included presence of peripheral or autonomic neuropathies from causes other than diabetes, conditions potentially responsible for autonomic dysfunction, severe comorbidities (such as malignancies, recent cardiovascular events, heart failure, advanced renal failure or liver disease), advanced peripheral arterial disease, severe psychiatric disorders or any other condition preventing understanding of the questionnaires. From 172 patients initially enrolled, 150 were included according to the selection criteria. The Ethics Committee of the University Hospital of Rome Tor Vergata approved the study. All participants provided written informed consent.

A complete clinical history was recorded regarding diabetes, comorbidity, cardiovascular disease and any potential cause of polyneuropathy. Height, weight, waist circumference, casual blood pressure (BP) and blood glucose at the moment of neurological assessment were measured.

A subject who smoked regularly at least one cigarette per day was considered a current smoker and alcohol consumption was recorded. Subjects who took part in leisure-related physical activity for at least 1 h per week were considered physically active.

Routine laboratory assessment was performed including HbA1c, cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, serum creatinine and 24-h urinary albumin excretion. Micro- and macroalbuminuria were considered present with a 24-h albumin excretion of 30–299 mg or ≥300 mg, respectively. The presence of non-proliferative or proliferative retinopathy was determined via ophthalmoscopic examination. Peripheral arterial disease was considered present on the basis of claudication and/or absence of palpable dorsalis pedis and/or posterior tibial pulses or instrumental reports (Doppler sonography and magnetic resonance angiography) - see Supplementary Tables S1 and S2 for patients' details.

2.2. Assessment of DPN and CAN

Neurological assessment included evaluation of neuropathic symptoms and deficits using the Questionnaire of the Michigan Neuropathy Screening Instrument (MNSI-Q) and the Michigan Diabetic Neuropathy Score (MDNS), respectively.[50] Vibration perception threshold (VPT) was measured using the Biothesiometer (Biomedical instruments, Newbury, OH, USA) at the hallux dorsum and at the lateral malleolus,[51] and age-related normal values derived from literature were used.[52] Cold (CTT) and warm thermal perception thresholds (WTT) were assessed using the Neuro Sensory Analyzer TSA-II (Medoc, Ramat Yishai, Israel) at the dorsum of both feet following the levels test procedure. The definition of DPN (probable) required the presence of at least two abnormalities among neuropathic symptoms, signs, vibration perception threshold, and thermal perception thresholds.[1] The *Douleur neuropathique en 4 questions* (DN4) was also used, as a validated screening tool for neuropathic pain.[53]

We performed four Cardiovascular autonomic reflex tests (CARTs), three based on heart rate response to deep breathing, lying to standing, and to Valsalva manoeuvre, and the orthostatic hypotension test, according to standard procedure and using age-related reference values.[53] An autonomic score was calculated by giving a score of 0 for a normal result, 1 for a borderline result and 2 for an abnormal result (range 0–8). We considered patients with ≥1 abnormal cardiovagal test as having early CAN and those with ≥2 abnormal tests as having confirmed CAN.[1,53]

2.3. DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood using standard procedures. Rs3746444 polymorphism located in the *MIR499A* gene was analyzed by allelic discrimination assay by TaqMan technology using MGB-specific allelic probes (Code c_2142612_30 by Life Technologies). In each run we used positive (samples previously confirmed by direct sequencing as heterozygous and/or variant homozygous) and negative controls. Moreover, *MIR499A* gene was amplified by PCR and analyzed by direct sequencing (ABI 3130xl Automated Sequencer [Applied Biosystems, Foster City, CA]) in 30 patients. The region amplified included the pre-miR region plus 200 bp upstream and downstream of flanking sequence. Primers sequences are the following: forward 5′-ACCAGGCCCCTTGTCTCTAT-3′ and reverse 5′-GAGACCCTTC GCTGTCTCC-3′.

2.4. Statistical analysis

The Hardy–Weinberg Equilibrium (HWE) was verified by the Pearson's Chi-squared test. Differences in genotypic frequencies between groups (CAN with or without, DPN with or without) were evaluated by the Pearson's chi-square test or by the Fisher's Exact test, when required. Odds ratios (OR) with 95% CI were calculated. The association analysis was performed according to additive, recessive and dominant model (Table 1). Clinical, metabolic and neurological parameters were subsequently evaluated according to *MIR499A* genotype in a recessive model (see Tables 2 and 3).

A binary regression analysis was used to evaluate the contribution of genetic (*MIR499A* SNP) and non-genetic factors (age, sex, disease duration, Hb1Ac, and BMI) to the neuropathy susceptibility. All statistical analyses were performed by SPSS program ver. 19 (IBM Corp, Armonk, NY, USA). Two-tailed P values <0.05 were considered statistically significant.

3. Results

3.1. Clinical characteristics of participants

We included 150 participants with type 2 diabetes (89 men), with a mean age of 63.8 ± 8.13 years, a diabetes duration of 12.64 ± 9.48 years, a body mass index (BMI) of 30.68 ± 5.21 kg/m^2, and HbA1c of 7.27 ± 1.48, among which 85.2% had hypertension, 32.4% micro- or macroalbuminuria, and 28% retinopathy (Supplementary Table S1).

According to neurological assessment and CARTs, 46.3% of participants were diagnosed as having DPN, 23.8% early CAN, and 9.8% confirmed CAN (Supplementary Table S2). The small discrepancies in the tables in the number of cases are due to the fact that some parameters (both clinical and neurological) were not available for all subjects.

3.2. Genetics association analysis

The rs3746444 A > G polymorphism in *MIR499A* gene was analyzed in the 150 participants with type 2 diabetes. The frequency of the G variant allele was 0.24, consistent with the frequency reported for the Tuscan population (23%) in the 1000 genome project. AA, AG, and GG genotypes were present in 56%, 38%, and 6% of patients, respectively. Deviation from Hardy–Weinberg equilibrium was not observed.

We performed a genotype-phenotype correlation analysis between the SNP and the presence of early CAN, confirmed CAN and DPN. Table 1 shows the results of these analyses: the association was evaluated under additive, dominant and recessive genetic models. We observed significant associations between CAN and *MIR499A* SNP with the recessive genetic model: the GG genotype was significantly more present in patients that developed early CAN (P = 0.006 and OR = 7.57) and confirmed CAN (P = 0.006 and OR = 9.92). These associations were confirmed also after corrections for age, BMI, disease duration, sex and HbA1c (P_{adj} = 0.002 and P_{adj} = 0.0005). Regarding DPN, the variant allele of the SNP showed a borderline association considering both the dominant and recessive model (P = 0.05 and P = 0.08). However, the association reached statistical significance after correction for age, BMI, disease duration sex and HbA1c only in the recessive model (P_{adj} = 0.038 and OR_{adj} = 6.56).

Considering that the most significant associations were seen with the recessive model, we evaluated the neurological parameters of patients according to rs3746444 genotypes and to a recessive model (Table 2). Patients carrying the GG genotype presented higher MDNS (P = 0.017), VPT (P = 0.01), percentage of abnormal thermal thresholds (P = 0.01) and CAN score (P < 0.001), and a lower number of correct answers to 10 g monofilament (P = 0.038) compared with patients carrying the other two genotypes (AA and AG).

Subsequently, we verified whether the GG genotype was correlated with any clinical or metabolic parameters. As shown in Table 3, there were no significant differences between patients carrying the GG genotype and those carrying the other two genotypes.

Finally, we performed two different multivariate analyses considering early CAN and DPN as dependent variables. We included in both models the rs3746444 GG genotype, age, BMI, sex, disease duration and HbA1c. These analyses confirmed that rs3746444 GG genotype, disease duration and HbA1c were risk factors for the development of CAN with an R^2 of 0.26. Regarding DPN, besides the contributions of rs3746444 GG genotype, disease duration and HbA1c, also age seemed to play a modest role, with a total R^2 of 0.21 (Table 4).

Lastly, we performed a linear multiple regression analysis to evaluate the contribution of rs3746444 GG genotype, age, BMI, sex, disease duration, HbA1c, physical activity, systolic BP, insulin treatment, LDL cholesterol, eGFR, and retinopathy to the definition of CAN score. This analysis confirmed that *MIR499A* GG genotype was the major factor contributing to CAN score (R^2 = 0.31) (Table 5).

Table 1
Associations between *MIR499A* SNP and the presence of diabetic neuropathy.

	MIR499A rs3746444 genotypes				Dominant model (AG + GG vs AA)				Recessive model (GG vs AA + AG)			
	AA	AG	GG		P	OR (95% CI)	P[a]	OR[a] (95% CI)	P	OR (95% CI)	P[a]	OR[a] (95% CI)
With early CAN (N = 34)	17	11	6	P = 0.008	0.42	1.37 (0.63–2.96)	0.59	1.28 (0.53–3.13)	**0.006**	7.57 (1.78–32.19)	**0.002**	16.08 (2.82–91.59)
Without early CAN (N = 109)	63	43	3									
With confirmed CAN (N = 14)	6	4	4	P = 0.001	0.30	1.79 (0.59–5.47)	0.33	1.92 (0.51–7.24)	**0.006**	9.92 (2.29–42.89)	**0.0005**	35.02 (4.77–257)
Without confirmed CAN (N = 124)	74	50	5									
With DPN (N = 69)	33	29	7	P = 0.052	**0.05**	1.92 (1.00–3.70)	0.06	2.04 (0.97–4.28)	0.082	4.40 (0.88–21.95)	**0.037**	6.56 (1.12–38.37)
Without DPN (N = 80)	51	27	2									

Significant associations are reported in bold. Small discrepancies in the total number of subjects for CAN and DPN are due to the fact that a complete neurological assessment was not available for all subjects.

[a] P and OR were adjusted for BMI, HbA1C, disease duration, sex and age.

Table 2
Neurological parameters of patients according to *MIR499A* SNP genotypes.

Parameters	*MIR499A* genotype			Recessive model
	AA (N = 84)	AG (N = 57)	GG (N = 9)	P
MNSI-Q	1.78 ± 2.16	2.78 ± 2.88	3.25 ± 2.44	0.24
MDNS	4.66 ± 4.13	5.62 ± 4.82	9 ± 5.37	**0.017**
Monofilament 10 g (number of correct answers)	8.86 ± 2.14	8.45 ± 1.93	7.06 ± 3.16	**0.038**
VPT hallux (Volt)	18.21 ± 9.99	20.68 ± 11.22	28.86 ± 12.76	**0.01**
CTT dorsal foot (°C)	29.4 ± 2.75	28.72 ± 5.08	26.99 ± 3.78	0.07
WTT dorsal foot (°C)	36.85 ± 3.92	37.29 ± 4.26	39.86 ± 2.57	0.15
With abnormal thermal thresholds N/total (%)	23/75 (31%)	18/46 (39%)	6/7 (86%)	**0.01**
DN4	1.23 ± 1.73	1.83 ± 2.15	1.87 ± 1.73	0.20
Expiration/inspiration ratio	1.24 ± 0.14	1.24 ± 0.10	1.17 ± 0.13	0.1
Lying to standing (30:15)	1.15 ± 0.09	1.17 ± 0.12	1.10 ± 0.15	0.09
Valsalva ratio	1.45 ± 0.29	1.44 ± 0.23	1.26 ± 0.16	0.06
Orthostatic hypotension (mm Hg)	9.76 ± 7.74	8.16 ± 7.74	14.7 ± 14.81	0.09
CAN score	0.99 ± 1.46	0.81 ± 1.47	3 ± 2.74	<**0.001**

MNSI-Q: Questionnaire of Michigan Neuropathy Screening Instrument; MDNS: Michigan Diabetic Neuropathy Score; VPT: vibration perception threshold; CTT: cold thermal threshold; WTT: warm thermal threshold; DN4: Douleur neuropathique en 4 questions.
Quantitative values are reported as the mean ± standard deviation. Qualitative values are reported as absolute number (over the total) and percentage (in parenthesis). Significant associations are reported in bold.

4. Discussion

Since their discovery, miRNAs have been extensively investigated due to their role in post-transcriptional regulation and involvement in pathological processes. Indeed, a growing number of studies has shown that miRNAs are dysregulated in various diseases and play key roles in many disease-related processes. Interestingly, some dysregulations in miRNAs have been described in animal models of neuropathic pain.[54–57] Recently, we have reported some polymorphisms in miRNA genes associated with both diabetes[58] and neuropathic complications.[18]

In the current paper, we have investigated the role of a polymorphism in *MIR499A* gene with respect to neuropathic complications in a small cohort of Italian people with type 2 diabetes. The rs3746444 SNP in the pre-miRNA region of *MIR499A* is a common variant and is strongly associated with many diseases, including several types of cancer,[32,59] autoimmune[49] and cardiovascular diseases.[60,61]

Our main result is the significant association between the GG genotype and the presence of CAN (both early and confirmed) and its severity, suggesting that the GG genotype confers a higher risk of developing this form of neuropathy. The association is confirmed also after adjustment for BMI, HbA1c, disease duration, sex and age. Notably, the GG genotype is also associated with several neurological parameters, which are markers of sensorimotor deficits (such as a higher value of MDNS, a lower number of correct answers to 10 g monofilament, a higher VPT, a higher percentage of abnormal thermal thresholds), and of autonomic impairment (i.e., a higher CAN score). Regarding DPN, we have observed a borderline association both with the dominant and recessive model. However, only the association of GG genotype achieves significance after correction for BMI, HbA1c, disease duration, sex, and age in a recessive model.

The rs3746444 polymorphism consists of an A > G nucleotide substitution that creates a mismatch in the stem loop of miR-499 precursor and it has been hypothesized that it could influence the maturation of both miR-499a-5p and miR-499a-3p and the binding of miR-499a-3p to its targets.[62]

Regarding the potential role of rs3746444 SNP in the pathogenesis of diabetic neuropathies we can only speculate. MiR-499 is known to be

Table 3
Anthropometric, clinical and metabolic parameters of patients according to *MIR499A* rs3746444 genotype.

	Rs3746444 genotype			P (GG vs AA + AG)
	AA	AG	GG	P
Subjects (men/women)	52/32	32/25	5/4	0.81
Age (years)	63.4 ± 7.7	64.6 ± 9.21	62.89 ± 3.66	0.72
Disease duration (years)	11.63 ± 8.83	14.51 ± 10.3	10.11 ± 8.82	0.41
BMI (kg/m^2)	30.4 ± 4.67	30.81 ± 5.48	32.41 ± 8.05	0.31
Insulin treated N/total (%)	21/84 (25%)	22/57 (38.6%)	5/9 (55.6%)	0.15
HbA1c (%)	7.34 ± 1.55	7.15 ± 1.38	7.48 ± 1.56	0.67
HbA1c (mmol/mol)	56.67 ± 18.86	54.61 ± 15.11	58.33 ± 17.28	0.66
Total cholesterol (mg/dl)	173.95 ± 35.62	168.82 ± 41.43	163.56 ± 60.09	0.54
HDL cholesterol (mg/dl)	47.75 ± 15.46	45.04 ± 10.69	42.67 ± 13.03	0.4
Triglycerides (mg/dl)	149.42 ± 107.91	177.95 ± 365.06	121.69 ± 29.47	0.63
Serum creatinine (mg/dl)	0.96 ± 0.27	0.94 ± 0.24	1.06 ± 0.26	0.24
eGFR (ml/min 1.73 m^2)	92.04 ± 29.11	90.88 ± 34.15	94.56 ± 60.10	0.79
With microalbuminuria N/total (%)	25/75 (33.3%)	15/49 (30.6%)	2/6 (33.3%)	1
Casual systolic blood pressure (mm Hg)	137.98 ± 20.17	136.84 ± 15.23	138.0 ± 19.99	0.94
Casual diastolic blood pressure (mm Hg)	78.67 ± 9.88	77.72 ± 17.22	74.44 ± 11.02	0.4
With hypertension N/total (%)	70/83 (84.3%)	50/57 (87.7%)	7/9 (77.8%)	0.62
With peripheral arterial disease N/total (%)	7/83 (8.4%)	7/56 (12.5%)	3/9 (33.3%)	0.07
With diabetic retinopathy N/total (%)	18/78 (23.1%)	16/50 (32%)	4/7 (57.1%)	0.1
With cardiovascular disease N/total (%)	18/84 (21.4%)	12/57 (21.1%)	1/9 (11.1%)	0.69
Current smokers N/total (%)	19/80 (23.8%)	5/55 (9.1%)	2/9 (22.2%)	0.74
Regular physical activity N/total (%)	53/78 (67.9%)	27/55 (49.1%)	4/9 (44.4%)	0.49
Alcohol consumption N/total (%)	26/69 (37.7%)	14/48 (29.2%)	2/8 (25%)	0.72

Quantitative values are reported as the mean ± standard deviation. Qualitative values are reported as absolute number (over the total) and percentage (in parenthesis). Small discrepancies in the numbers of cases are due to the fact that some parameters were not available for all subjects.

Table 4
Logistic regression analysis with cardiovascular autonomic neuropathy (CAN) and diabetic polyneuropathy (DPN) as dependent variables.

Variables	Early CAN ($R^2 = 0.26$)		DPN ($R^2 = 0.21$)	
	P	OR (95%CI)	P	OR (95%CI)
MIR499A (GG vs AA + AG)	**0.002**	16.08 (2.82–91.59)	**0.037**	6.56 (1.12–38.37)
Age (years)	0.11	0.95 (0.89–1.01)	**0.05**	1.05 (1–1.11)
Sex (male)	0.16	0.35 (0.18–1.33)	0.52	0.78 (0.36–1.68)
Diseases duration (years)	<**0.001**	1.12 (1.06–1.17)	**0.003**	1.08 (1.03–1.13)
BMI (kg/m^2)	0.91	1.00 (0.92–1.10)	0.11	1.07 (0.99–1.16)
HbA1c (%)	**0.046**	1.40 (1.00–1.94)	**0.04**	1.34 (1.01–1.78)

Significant results are reported in bold.

preferentially expressed in cardiac tissue, but has recently been observed in a diabetic rat model also in nucleus ambiguus (which hosts most preganglionic cardiovagal neurons)[63,64]; the association of *MIR499*A A/G rs3746444 SNP with ischemic stroke and post-myocardial infarction prognosis has also been described.[11,45] The link between miR-499 and cardiovascular disease could be its inhibitory regulation of the apoptotic pathway involving calcineurin (CnA) and dynamin-related protein (DRP1). CnA dephosphorylates DRP1, which causes mitochondrial fission and cell apoptosis. In animal models of diabetic neuropathy, DRP1 induces increase in cell dysfunction (mitochondrial fission) in response to hyperglycemia, whereas in vitro DRP1 inhibition reduces hyperglycemia-dependent cell dysfunction. MiR-499 overexpression inhibits CnA and CnA cellular activity and attenuates the effect of DRP1 and prevents apoptosis.[65–68] Interestingly, mitochondrial dynamics are important in neuronal development and their perturbations have been observed in major neurodegenerative disorders.[69] Moreover, mitochondrial fission has been seen to accompany ROS production and oxidative damage in response to a variety of neuronal insults.[70] Therefore, in light of all these considerations, in a future perspective, it would be interesting to evaluate whether there is a link between mitochondrial copy number and *MIR499*A genotypes in patients with neuropathy.

Moreover, Wang et al.,[47] investigating the baroreceptor reflex, observed that miR-499 expression was significantly increased in heart and in nucleus ambiguus of diabetic rats. Its over-expression was able to inhibit GADD45a mRNA that, in turn, seems to be activated after cellular stresses and stimuli, including DNA damage, cellular senescence, apoptosis, oxidants and others.[71] However, in accordance with Vinci et al.[72] it seems that the GG genotype causes a reduced miRNA expression in comparison with GA and AA genotypes; although this expression reduction has been seen in colorectal cancer patients, we might argue that the inhibition of GADD45a function is not a likely mechanism linking GG genotype with susceptibility to diabetic neuropathy. On the other hand, a reduced miR-499-5p level has been observed in hepatic insulin resistance by Wang et al.[46]: they reported that miR-499-5p was involved in the signaling pathway of IRS1/PI3K/AKT and in particular miR-499-5p seems to target PTEN, which is an important regulator of the insulin signaling pathway.[73] Therefore, we can speculate that the GG genotype causes reduced miR-499-5p levels, and as a consequence an increase in PTEN signal and impairing the insulin signaling.[46] Finally, *MIR499A* can affect the inflammatory reactions through modulating C-reactive protein,[61] and inflammation is one of the main pathogenic mechanisms of DPN and CAN.[65,74] However, also in this case the pathogenic meaning of a hypothetical association between *MIR499A* GG genotype and inflammation (i.e., inhibition or promotion of inflammation) remains elusive.

Table 5
Multiple linear regression analysis for CAN score as dependent variable.

Independent variables	B standardised coefficient	t-Statistics	P	R^2 (R^2adj)
Sex (male)	−0.12	−1.32	0.19	0.35
Age (years)	0.07	0.61	0.54	(0.27)
BMI (kg/m^2)	0.18	1.81	0.07	
Physical activity (with)	0.07	0.88	0.38	
Duration (years)	0.25	2.14	**0.035**	
HbA1c (%)	−0.01	−0.09	0.93	
Insulin dose (units/kg)	0.18	1.62	0.11	
Systolic BP (mm Hg)	−0.08	−0.87	0.39	
LDL cholesterol (mg/dl)	0.02	0.19	0.85	
eGFR (ml/min/1.73m^2)	0.11	0.1	0.32	
Retinopathy (with)	0.13	1.29	0.20	
MIR499A (GG vs AA + AG)	0.29	3.39	**0.001**	

Significant results are reported in bold.

The finding of a stronger association of *MIR499*A gene polymorphism with CAN compared to DPN might be interpreted with regard to pathogenetic differences, to autonomic testing, and to miR-499 expression in different tissues. Although DPN and CAN share the main pathogenetic mechanisms and may develop in parallel, no complete clinical overlapping exists between them, and the possibility arises that the GG genotype may increase susceptibility to a pathogenetic mechanism more active in CAN than in DPN, which however remains undefined. Since miR-499 is now considered a biomarker of cardiovascular events[45] and cardiac disease may exert a confounding effect on cardiovascular autonomic testing,[53] the association might merely depend on a preferential coexistence of CAN with cardiovascular disease in the group with GG genotype. However, an association between GG genotype and cardiovascular disease was not documented in this study (Table 3). MiR-499 is primarily expressed in the heart and in the nucleus ambiguus, which are both in the autonomic cardiovascular control circuit as the target organ (with blood vessels) and a central hub of the autonomic network,[64] respectively. Thus, a change in miR-499 expression, possibly consequent to GG genotype, might impair protective mechanisms in those areas and be more easily associated with risk of CAN compared to DPN.

Although our results are promising and might be useful in identifying patients more prone to develop diabetic neuropathies, they should be considered as preliminary ones and a replication study is necessary. In fact, the sample size of our study constitutes a limitation: especially in the case of CAN, just a small number of subjects had one or two abnormal tests. However, it is worth highlighting that our sample is well characterized for neurological parameters and that the diagnostic assessment of clinical and instrumental neurological measures was carried out in an accurate way and according to current guidelines. Using an online statistical power calculator (OpenEpi, version 2013), and considering the frequency of homo-variant genotype, the power of our study was 82% (for confirmed CAN) and 77% (for early CAN). We considered these power analysis results adequate to support the hypothesis that GG genotype could play a role in neuropathy development.

In conclusion, we describe for the first time an association between a common variant in *MIR499A* gene and diabetic neuropathies. In particular, the most interesting finding is the high risk of developing CAN along with a more severe form of CAN in diabetic patients carrying the rs3746444 GG genotype, suggesting a role of miR-499 in cardiovascular autonomic dysfunction in diabetes. Of course, this data needs to be replicated in a larger cohort of patients, and in fact we are planning to extend the population of the study. Moreover, functional studies will be necessary to investigate whether and how miR-499 might influence key pathways involved in the pathogenesis of diabetic neuropathy and/or in autonomic dysfunction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdiacomp.2017.10.011.

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