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Trained breathing-induced oxygenation acutely reverses cardiovascular autonomic dysfunction in patients with type 2 diabetes and renal disease

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Abstract

Aims Cardiovascular autonomic dysfunction, evaluated as baroreflex sensitivity (BRS), could be acutely corrected by slow breathing or oxygen administration in patients with type 1 diabetes, thus suggesting a functional component of the disorder. We tested this hypothesis in patients with the type 2 diabetes with or without renal impairment.

Methods Twenty-six patients with type 2 diabetes (aged 61.0 ± 0.8 years, mean $\pm$ SEM; duration of diabetes 10.5 ± 2 years, BMI 29.9 ± 0.7 kg/m², GFR 68.1 ± 5.6 ml/min) and 24 healthy controls (aged 58.5 ± 1.0 years) were studied. BRS was obtained from recordings of RR interval and systolic blood pressure fluctuations during spontaneous and during slow, deep (6 breaths/min) controlled breathing in conditions of normoxia or hyperoxia (5 l/min oxygen).

Results During spontaneous breathing, diabetic patients had lower RR interval and lower BRS compared with the control subjects (7.1 ± 1.2 vs. 12.6 ± 2.0 ms/mmHg,

$p < 0.025$). Deep breathing and oxygen administration significantly increased arterial saturation, reduced RR interval and increased BRS in both groups (to 9.6 ± 1.8 and 15.4 ± 2.4 ms/mmHg, respectively, $p < 0.05$, hyperoxia vs. normoxia). Twelve diabetic patients affected by chronic diabetic kidney disease (DKD) presented a significant improvement in the BRS during slow breathing and hyperoxia ($p < 0.025$ vs. spontaneous breathing during normoxia).

Conclusions Autonomic dysfunction present in patients with type 2 diabetes can be partially reversed by slow breathing, suggesting a functional role of hypoxia, also in patients with DKD. Interventions known to relieve tissue hypoxia and improve autonomic function, like physical activity, may be useful in the prevention and management of complications in patients with diabetes.

Keywords Baroreflex sensitivity · Cardiovascular autonomic neuropathy · Diabetic kidney disease · Hypoxia · Oxygen · Type 2 diabetes mellitus

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Introduction

Dysfunction of the autonomic nervous system, resulting in sympathetic overactivity, is common in clinical conditions at high risk of cardiovascular disease, such as diabetes, hypertension, obesity and chronic kidney disease (CKD) [1–4].

In particular, in patients with diabetes excess sympathetic output may lead to impaired autonomic regulation of the cardiovascular activity that causes tachycardia at rest, exercise intolerance and decreased baroreflex sensitivity (BRS) [5].

It is well established that autonomic dysfunction plays an important role in the pathogenesis of cardiovascular

disease (CVD) and is also a predictor of survival in patients with diabetes [6–8]. In addition to being a direct culprit for cardiovascular disease, the autonomic dysfunction further accelerates the progression of diabetic kidney disease (DKD), presently the most prevalent cause of end-stage renal failure, and represents a powerful booster of cardiovascular morbidity and mortality in CKD patients [9–11]. Furthermore, by accelerating the renal injury, autonomic dysfunction fuels itself because renal failure is also an independent trigger of sympathetic activation [12].

While there is sound evidence that autonomic dysfunction is a determinant of cardiovascular risk, the reasons why the sympathetic nervous system is activated in diabetes are poorly understood. There is a general belief that a wide spectrum of vascular, metabolic and toxic injuries, including alterations in the endoneural circulation, activation of the polyol pathway, accumulation of advanced glycation end products and inflammation, may result in neuronal damage [13–16]. Moreover, this complex picture could be further aggravated by uraemic milieu that is a unique mixing of metabolic, hemodynamic and nutritional disorders, which may all participate in the development of neuropathy [17].

However, the possibility that this chain of events can be preceded or associated with some degree of functional autonomic involvement has been recently proposed and found in patients with type 1 diabetes [18], but has so far never been tested in type 2 diabetes, and particularly in subjects with some degree of renal involvement.

This is a crucial point since whether the autonomic dysfunction is the expression of neural structural damage, i.e. an irreversible disorder, or a mainly functional and reversible condition also has practical implications. The latter concept is supported by evidence that interventions based on exercise training have beneficial effects on the autonomic regulation [19, 20]. Moreover, it has been demonstrated that hyperoxia in patients with type I diabetes improves the baroreflex reactivity [18].

Taken together, these data indicate a functional autonomic disorder, paving the way for a paradigm shift, suggesting that autonomic dysfunction is potentially treatable. In order to test this hypothesis and in particular the role of hypoxia, we evaluated the effects of respiratory manoeuvres and short-term oxygen administration on the BRS in healthy subjects and patients with type 2 diabetes with and without renal impairment.

Methods

Study design

The study was carried out in patients with type 2 diabetes from the outpatient clinic for diabetes, hypertension and

renal diseases at San Matteo Hospital of Pavia, Italy. We enrolled patients aged 18–70 years and collected data on anthropometric parameters (body weight and body mass index—BMI), personal and family history, duration of diabetes, current medications, insulin doses and smoking.

Resting systolic and diastolic blood pressures (SBP and DBP, respectively) were measured twice, while the subjects were seated. The diagnosis of hypertension was made if the mean of two readings was $>140/90$ mmHg [21]. Exclusion criteria were as follows: BMI >35 kg/m² and the occurrence of metabolic complications or cardiovascular events during the last 3 months. Moreover, in order to evaluate the impact of renal function on autonomic parameters, we compared diabetic patients with DKD, defined as the presence of microalbuminuria or macroalbuminuria and/or estimated GFR values ≤ 60 ml/min for a period of at least 3 months, with diabetic patients without DKD [22]. Healthy subjects from our staff were enrolled as controls. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Foundation IRCCS Policlinico “San Matteo” of Pavia, Italy, and written informed consent was obtained from each participant prior to enrolment in the study.

Experimental protocol

All participants were investigated in a quiet room, at a temperature between 19 °C and 23 °C, between 0800 and 1400 hours. The participants received instructions to refrain from alcohol for 36 h, and caffeinated beverages and cigarettes for 12 h prior to the examination. A light meal was permitted 2 h before testing. If a participant reported or measured symptoms or values of hypoglycaemia in the previous 24 h, the test was postponed. ECG was recorded using a bipolar precordial lead. We monitored continuous blood pressure, with a CNAP digital plethysmograph (CNSystem, Graz, Austria) from the middle finger of the right arm held at heart level, and pulse oximetry (Cosmo; Novamatrix, Wallingford, CT, USA). The signals were simultaneously recorded in the supine position during 5 min spontaneous breathing and during 2 min slow deep breathing at the rate of six cycles/min. Subsequently, the participants repeated the entire protocol while breathing 5 l/min oxygen. Signal recordings started after the first 5 min of oxygen administration to allow stabilisation of oxygen saturation and ventilation. All signals were simultaneously acquired on a personal computer with an analogue-to-digital converter with a 12-bit resolution at a sampling rate of 400 Hz.

BRS assessment

From the original data, time series of the RR interval and SBP were obtained. Previous studies have shown a poor

correlation between different indices of BRS, while on the other hand, no method has shown clear superior performance over the other [23]. Accordingly, we computed a set of seven different tests and used their average [24].

BRS was determined from spontaneous fluctuations in the RR interval and SBP during the spontaneous and 6/min recordings using the positive and negative sequence methods [25], the alpha coefficient in the low- and high-frequency bands and its average [26], and the transfer function technique [27]. In the sequence methods, BRS was estimated by identifying spontaneously occurring sequences of three or more consecutive heartbeats in which both the SBP and the subsequent RR intervals changed in the same direction. The minimum criteria for change were 1 mmHg for SBP and 5 ms for the RR intervals. For positive and negative sequences with a correlation coefficient between the RR intervals and the SBP exceeding 0.85, the regression slopes were calculated, and the average was taken as a measure of BRS positive and negative slopes, respectively. The other four BRS methods were calculated by autoregressive univariate and bivariate spectral analysis [28]. The alpha coefficient was calculated as the square root of the ratio of the powers of RR intervals and SBP in the low-frequency range (0.04–0.15 Hz) and in the respiratory (0.15–0.40 Hz) high-frequency range when coherence was >0.5 , and the phase difference between the SBP and RR intervals was negative. In the transfer function method, BRS was calculated as the average value of SBP–RR cross-spectrum divided by the SBP spectrum in the low-frequency range (0.04–0.15 Hz), when coherence exceeded 0.5.

Cardiovascular autonomic neuropathy (CAN) assessment

The presence of CAN was assessed using cardiovascular reflex tests as defined by a previous published article [29]. Each autonomic index was compared to the 90 percentile of age-related normal ranges obtained with linear regression equations from an earlier published study [30]. Every abnormal finding gave the subject one point of autonomic functional score. Definite CAN was diagnosed with the presence of 2 or more abnormal findings.

Laboratory tests

HbA1c concentrations were determined by an immunoturbidimetric immunoassay, while serum lipids and creatinine were measured by enzymatic methods. The CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation was used for GFR estimation [31]. Proteinuria was evaluated as urinary albumin excretion rate (AER) measured from 24-h urine collections. Normal AER was

defined as values persistently <30 mg/24 h, microalbuminuria as $AER \geq 30 < 300$ mg/24 h and macroalbuminuria as $AER \geq 300$ mg/24 h in at least two out of three urine collections [32].

Statistical analyses

Quantitative variables were represented by mean and Standard Error of Mean ($\pm$ SEM) and qualitative ones by number and percentage. Qualitative data were compared by means of Chi-square test or Fisher exact test for categorical variables or by means of unpaired Student's *t* test or nonparametric Mann–Whitney test in case of quantitative variables. Comparisons between conditions (normoxia vs. hyperoxia), breathing patterns (spontaneous vs. 6 breaths/min) and participant group (healthy control/diabetic) were made by means of a three-level mixed-design analysis of variance (factorial design to test differences between different groups of participants, and repeated measures on two factors to test the differences induced by breathing patterns (spontaneous vs. slow breathing) and by oxygen (normoxia vs. hyperoxia). All tests were two-sided. Associations between variables were assessed by fitting linear regression model. $p < 0.05$ was considered statistically significant.

Results

Participant characteristics

We enrolled 26 diabetic patients and 24 healthy subjects as controls. Their clinical characteristics are reported in Table 1.

As compared to the healthy control subjects, the diabetic patients presented a significantly higher BMI (29.9 ± 0.7 vs. 23.1 ± 0.7 kg/m², $p < 0.001$), higher levels of proteinuria and reduced renal function, with lower mean eGFR (68.1 ± 5.6 vs. 86.3 ± 3.6 ml/min, $p < 0.05$). Duration of diabetes was 10.5 ± 2 years, 24 diabetic patients (92 %) were taking antihypertensive drugs, while 6 patients presented microalbuminuria and 5 macroalbuminuria. Based on the presence of albuminuria and/or a reduced GFR, we divided the diabetic participants in nephropathic ($N = 12$, DKD+ subgroup) and non-nephropathic ($N = 14$, DKD– subgroup). The true cause of DKD (nephrosclerosis, diabetic nephropathy or other) was unknown but in two patients in whom renal biopsy showed a histological picture of diabetic nephropathy. DKD patients compared with non-DKD subjects had longer duration of diabetes and poorer glycaemic control. Their clinical data are shown in Table 2.

Table 1 Clinical characteristics and laboratory measurements of study participants (mean $\pm$ SEM)

	Diabetic patients	Controls	<i>p</i> value
<i>N</i>	26	24	–
Gender (M/F)	20/6	7/17	0.001
Age (years)	61.0 $\pm$ 0.8	58.5 $\pm$ 1.0	0.06
Duration of diabetes (years)	10.5 $\pm$ 2	0	–
BMI (kg/m ²)	29.9 $\pm$ 0.7	23.1 $\pm$ 0.7	<0.001
Current smokers, <i>n</i> (%)	7 (27)	2 (8)	0.14
Antihypertensive treatment, <i>n</i> (%)	24 (92)	0	–
ACE inhibitors	16	0	–
Calcium antagonists	8	0	–
AT-2r Blockers	13	0	–
Beta blockers ^a	10	0	–
Diuretics	13	0	–
statins	19	0	–
Insulin treatment, <i>n</i> (%)	7 (27)	0	–
Laser-treated retinopathy, <i>n</i> (%)	4 (15)	0	–
Microalbuminuria, <i>n</i> (%)	6 (23)	0	–
Macroalbuminuria, <i>n</i> (%)	5 (19)	0	–
HbA _{1c} (%)	6.7 $\pm$ 0.2	5.3 $\pm$ 0.2	0.004
mmol/mol	50 $\pm$ 1.9	34 $\pm$ 1.9	
Fasting blood glucose (mg/dl)	124.5 $\pm$ 5.0	83.8 $\pm$ 2.4	0.0001
(mmol/L)	6.9 $\pm$ 0.3	4.6 $\pm$ 0.1	
Total cholesterol (mg/dl)	176.2 $\pm$ 7.9	179.9 $\pm$ 14	0.43
HDL cholesterol (mg/dl)	46.8 $\pm$ 2.1	64.4 $\pm$ 6.3	0.018
Triacylglycerol (mg/dl)	143.2 $\pm$ 10.7	94.9 $\pm$ 8.5	0.0015
AER (mg/24 h)	495.4 $\pm$ 218	11.8 $\pm$ 1.8	0.018
eGFR (ml/min)	68.1 $\pm$ 5.6	86.3 $\pm$ 3.6	0.026
Serum creatinine (mg/dl)	1.32 $\pm$ 0.15	0.82 $\pm$ 0.05	0.016
Office SBP (mmHg)	135.6 $\pm$ 3.0	127.9 $\pm$ 3.8	0.11
Office DBP (mmHg)	80.1 $\pm$ 1.9	75.9 $\pm$ 1.8	0.12
Office heart rate (bpm)	74.8 $\pm$ 2.8	65.9 $\pm$ 1.5	0.01
Autonomic score	1.0 $\pm$ 0.2	0.4 $\pm$ 0.1	0.018

BMI Body mass index, *eGFR* estimated glomerular filtration rate, *AER* albumin excretion rate, *SBP* systolic blood pressure, *DBP* diastolic blood pressure

^a Discontinued 1 week before the study

Resting data and effect of slow breathing

During spontaneous breathing, diabetic patients presented with lower BRS values ($p < 0.025$) compared with the control participants. Other variables, including resting systolic and diastolic BP and oxygen saturation, were similar to controls (Table 3).

Deep controlled breathing at 6 breaths/min in normoxia induced in both the groups a significant increase in oxygen saturation ($p < 0.001$ vs. spontaneous breathing). The ANOVA also showed a significant ($p < 0.025$) interaction between groups and breathing pattern, indicating that in type 2 diabetic patients, the effect of slow deep breathing was slightly less efficient in increasing oxygen saturation

than in healthy controls. Also BRS increased in response to slow deep breathing, as compared to spontaneous breathing in both groups (effect of breathing pattern: $p < 0.01$ on ANOVA). The extent of the increase in BRS induced by slow breathing was similar in both healthy controls and type 2 diabetic patients (no significant interaction between groups and breathing pattern was found on ANOVA). Slow breathing induced a significant ($p < 0.05$ on ANOVA) reduction in diastolic blood pressure in both groups, and also a slight reduction in systolic blood pressure, though the latter did not reach statistical significance. The extent of this decrease was not influenced by oxygen administration (no interaction between breathing pattern and oxygen on ANOVA). Neither duration of diabetes, nor BMI, and the

Table 2 Clinical characteristics and laboratory measurements of diabetic patients with (DKD+) or without (DKD−) chronic kidney disease (mean $\pm$ SEM)

	DKD+	DKD−	<i>p</i> value
<i>N</i>	12	14	
Gender (M/F)	11/1	9/5	0.17
Age (years)	62.1 $\pm$ 1.2	60.9 $\pm$ 1.2	0.47
Duration of diabetes (years)	15.4 $\pm$ 3.6	6.3 $\pm$ 1.1	0.015
BMI (kg/m ²)	30.3 $\pm$ 0.9	29.7 $\pm$ 1.2	0.71
Current smokers, <i>n</i> (%)	2 (16)	5 (33)	0.39
Antihypertensive treatment, <i>n</i> (%)	12 (100)	12 (85)	1
ACE inhibitors	8	8	–
Calcium antagonists	4	4	–
AT-2r Blockers	7	6	–
Beta blockers ^a	5	5	–
Diuretics	8	5	–
Statins	9	10	–
Insulin treatment, <i>n</i> (%)	4 (33)	3 (21)	1
HbA _{1c} (%)	7.3 $\pm$ 0.3	6.2 $\pm$ 0.2	0.004
mmol/mol	56 $\pm$ 1.9	44 $\pm$ 1.9	
Fasting blood glucose (mg/dl)	126.1 $\pm$ 9.3	123.1 $\pm$ 5.2	0.77
(mmol/L)	7.0 $\pm$ 0.5	6.8 $\pm$ 0.3	
Total cholesterol (mg/dl)	177.0 $\pm$ 15.8	175.6 $\pm$ 7.4	0.93
HDL cholesterol (mg/dl)	43.7 $\pm$ 2.6	49.0 $\pm$ 3.1	0.24
Triacylglycerol (mg/dl)	170.2 $\pm$ 17.5	122.0 $\pm$ 10.8	0.022
Microalbuminuria (mg/24 h)	912.8 $\pm$ 387.4	15.4 $\pm$ 2.9	0.004
eGFR (ml/min)	40.7 $\pm$ 3.9	91.6 $\pm$ 2.8	0.001
Serum creatinine (mg/dl)	1.92 $\pm$ 0.21	0.81 $\pm$ 0.04	0.0001
Office SBP (mmHg)	131.2 $\pm$ 5.3	139.3 $\pm$ 3.2	0.19
Office DBP (mmHg)	77.5 $\pm$ 3.2	82.3 $\pm$ 3.2	0.22
Office heart rate (bpm)	76.7 $\pm$ 4.8	71.7 $\pm$ 3.1	0.46
Autonomic score	0.8 $\pm$ 0.3	1.1 $\pm$ 0.2	0.48

For definition of DKD, see “Methods”

DKD Diabetic kidney disease, BMI body mass index, eGFR estimated glomerular filtration rate, SBP systolic blood pressure, DBP diastolic blood pressure

^a Discontinued 1 week before the study

presence of microalbuminuria/macrob albuminuria were associated with autonomic function parameters.

Effect of oxygen inhalation

Inhalation of 5 l/min oxygen increased oxygen saturation during spontaneous breathing in both groups (Table 3). A similar increase was seen also during slow breathing, but the extent of the increase was limited by the fact that slow breathing alone also increased oxygen saturation. The ANOVA also showed a significant ($p < 0.05$) interaction between groups and oxygen, indicating that in type 2 diabetic patients, the effect of 5 l/min oxygen was slightly less efficient in increasing oxygen saturation than in healthy controls. Nevertheless, oxygen inhalation

significantly reduced heart rate (increase in RR interval) in both groups, and hyperoxia increased BRS during spontaneous breathing more in type 2 diabetic patients ($p < 0.025$) than in controls (p:ns). Oxygen slightly increased BRS also during slow breathing, but the lack of significance was likely due to the fact that slow breathing alone was already capable of significantly increase BRS.

The ANOVA showed that neither SBP nor DBP was affected by oxygen administration in our participants.

The BRS values obtained during spontaneous breathing in the diabetic patients approximately predicted the BRS increase induced by oxygen administration ($r = 0.512$, $p = 0.0075$) and showed a reverse effect for BRS values >12 ms/mmHg (calculated with the present methodology)

Table 3 Effects of oxygen and respiratory patterns on cardiorespiratory variables (mean $\pm$ SEM)

	Spontaneous breathing		6 breaths/min controlled breathing	
	Normoxia	Hyperoxia	Normoxia	Hyperoxia
<i>Baroreflex sensitivity (ms/mmHg)</i>				
Diabetic patients	7.1 $\pm$ 1.2	9.2 $\pm$ 1.0*	9.6 $\pm$ 1.8	10.8 $\pm$ 2.2
Controls	12.6 $\pm$ 2.0	13.0 $\pm$ 1.9	15.4 $\pm$ 2.4	15.4 $\pm$ 2.1
<i>p</i>	0.021	0.070	0.057	0.140
<i>Difference between groups: $p < 0.05$</i>				
<i>Influence of breathing pattern: $p < 0.01$</i>				
<i>*$p < 0.025$ versus normoxia</i>				
<i>RR interval (msec)</i>				
Diabetic patients	815 $\pm$ 30	873 $\pm$ 33	802 $\pm$ 29	830 $\pm$ 32
Controls	884 $\pm$ 21	956 $\pm$ 23	846 $\pm$ 21	911 $\pm$ 24
<i>p</i>	0.070	0.050	0.237	0.055
<i>Influence of oxygen: $p < 0.0001$</i>				
<i>Interaction group $\times$ oxygen: $p < 0.05$</i>				
<i>Oxygen saturation (%)</i>				
Diabetic patients	96.1 $\pm$ 0.3	98.5 $\pm$ 0.2	98.0 $\pm$ 0.2	99.0 $\pm$ 0.1
Controls	96.4 $\pm$ 0.3	99.0 $\pm$ 0.1	98.4 $\pm$ 0.2	99.3 $\pm$ 0.1
<i>p</i>	0.531	0.049	0.26	0.219
<i>Influence of oxygen: $p < 0.0001$</i>				
<i>Influence of breathing pattern: $p < 0.0001$</i>				
<i>Interaction group $\times$ oxygen: $p < 0.05$</i>				
<i>Interaction group $\times$ breathing pattern: $p < 0.025$</i>				
<i>Interaction breathing pattern $\times$ oxygen: $p < 0.0001$</i>				
<i>Systolic blood pressure (mmHg)</i>				
Diabetic patients	121.5 $\pm$ 3.2	120.3 $\pm$ 3.6	118.6 $\pm$ 3.5	118.4 $\pm$ 3.9
Controls	116.0 $\pm$ 3.1	117.3 $\pm$ 3.4	115.2 $\pm$ 3.0	115.9 $\pm$ 2.6
<i>p</i>	0.222	0.554	0.483	0.605
<i>Diastolic blood pressure (mmHg)</i>				
Diabetic patients	75.7 $\pm$ 1.8	76.0 $\pm$ 2.1	72.8 $\pm$ 2.0	73.8 $\pm$ 2.4
Controls	74.8 $\pm$ 2.0	75.5 $\pm$ 2.0	73.1 $\pm$ 2.1	74.5 $\pm$ 2.1
<i>p</i>	0.733	0.875	0.980	0.829
<i>Influence of breathing pattern: $p < 0.05$</i>				

from which oxygen administration reduced BRS. The same value was found in the control group.

The BRS response to oxygen was not influenced by glycaemic control or albuminuria.

Diabetic patients with renal impairment

The two subgroups of our diabetic participants showed similar responses to oxygen and slow breathing. Accordingly, after repeating the ANOVA, we found no significant effects of the groups on the variables measured, whereas the effects of the breathing pattern and oxygen remained evident, and similar in the two subgroups (Table 4), despite the small number in each subgroup. We found a significant inverse correlation between diabetes duration and eGFR values ($r = 0.453$, $p = 0.020$).

Discussion

Our data demonstrate that the autonomic dysfunction present in type 2 diabetic patients is significantly reversed by two different procedures that increase blood oxygenation, i.e. oxygen administration and a controlled trained method of breathing. Furthermore, we show that these manoeuvres are effective also in patients with diabetes and DKD. Since the immediate effect of the manoeuvres was an increase in arterial blood oxygen partial pressure, it can be assumed that their effects on autonomic dysfunction were secondary to an increase in tissue oxygen delivery.

In particular, two findings of our study deserve attention: (1) the evidence that hypoxia correction induced BRS improvements also in diabetic patients with DKD and (2) the demonstration that the response to oxygen and slow

Table 4 Effects of oxygen and respiratory patterns on cardiorespiratory variables in nephropathic (DKD+) and non-nephropathic (DKD−) diabetic patients (mean $\pm$ SEM)

	Spontaneous breathing		6 breaths/min controlled breathing	
	Normoxia	Hyperoxia	Normoxia	Hyperoxia
<i>Baroreflex sensitivity (ms/mmHg)</i>				
DKD+	7.4 $\pm$ 2.2	8.3 $\pm$ 1.5	10.5 $\pm$ 3.6	14.2 $\pm$ 4.6**
DKD−	6.8 $\pm$ 1.2	9.8 $\pm$ 1.6**	8.9 $\pm$ 1.6	7.8 $\pm$ 1.0
<i>p</i>	0.796	0.493	0.677	0.155
** <i>p</i> < 0.025 versus normoxia				
<i>RR interval (msec)</i>				
DKD+	806 $\pm$ 51	856 $\pm$ 55	801 $\pm$ 52	822 $\pm$ 56
DKD−	823 $\pm$ 35	886 $\pm$ 42	803 $\pm$ 34	836 $\pm$ 37
<i>p</i>	0.787	0.670	0.975	0.837
Influence of oxygen: <i>p</i> < 0.001				
Influence of breathing pattern: <i>p</i> < 0.01				
Interaction breathing pattern $\times$ oxygen: <i>p</i> < 0.001				
<i>Oxygen saturation (%)</i>				
DKD+	96.4 $\pm$ 0.5	98.4 $\pm$ 0.4	98.1 $\pm$ 0.3	98.8 $\pm$ 0.2*
DKD−	96.0 $\pm$ 0.3	98.5 $\pm$ 0.3	98.0 $\pm$ 0.3	99.2 $\pm$ 0.1
<i>p</i>	0.438	0.715	0.714	0.087
Influence of oxygen or breathing pattern: <i>p</i> < 0.001				
Interaction breathing pattern $\times$ oxygen: <i>p</i> < 0.001 * <i>p</i> < 0.05 versus normoxia				
<i>Systolic blood pressure (mmHg)</i>				
DKD+	127.8 $\pm$ 5.7	126.5 $\pm$ 6.9	123.5 $\pm$ 6.5	126.5 $\pm$ 6.9
DKD−	116.1 $\pm$ 2.9	114.9 $\pm$ 3.3	114.3 $\pm$ 3.3	114.9 $\pm$ 2.6
<i>p</i>	0.069	0.199	0.199	0.108
<i>Diastolic blood pressure (mmHg)</i>				
DKD+	78.8 $\pm$ 2.8	78.9 $\pm$ 3.3	76.0 $\pm$ 3.6	76.5 $\pm$ 3.3
DKD−	73.1 $\pm$ 2.0	73.5 $\pm$ 2.6	70.4 $\pm$ 2.10	71.5 $\pm$ 3.4
<i>p</i>	0.115	0.210	0.170	0.306

DKD Diabetic kidney disease

breathing was independent of the duration of diabetes and its metabolic control, two factors associated with the probability of developing neuronal damage in diabetes.

Of note we found that in both groups of patients (DKD− and DKD+) slow breathing and oxygen administration improved BRS compared to spontaneous breathing in normoxia that was considered the basal condition. However, these improvements reached statistical significance only during spontaneous breathing and hyperoxia for DKD− patients and during slow breathing and hyperoxia for DKD.

These differences could be, at least, due to the small number of patients in each subgroup of diabetic subjects. On the other hand, it is possible that DKD patients present a more severe hypoxia-dependent autonomic dysfunction that requires the achievement of higher oxygen saturation to be corrected (as also evident in Table 4 in DKD+ patients, only hyperoxia during slow breathing was associated with significant increased oxygen saturation).

Actually, multiple factors have been claimed to contribute to the pathogenesis of autonomic dysfunction in DKD, including activation of the SNS by afferent signals, vascular abnormalities and activation of sympathetic nervous discharge by renal ischaemic disease, but none of these mechanisms has been proved by sound experimental results, nor have they been translated into effective therapeutic interventions [33–35]. In particular, invasive procedures such as renal denervation and baroreceptor-activating therapy have been proposed based on the idea that afferent neuronal signals, from the kidneys or carotid baroreceptors play a role in stimulating sympathetic activity [36, 37]. In previous studies, these procedures were effective in reducing blood pressure and SNS overdrive in patients with resistant hypertension [38, 39], but a recent controlled investigation carried out in a large patient population did not confirm the therapeutic validity of the procedure [40].

Pharmacological treatments, including beta blockers and RAAS blockade (with ACE inhibitor (ACEi) or

angiotensin II receptor blocker (ARB), cause a reduction in all-cause mortality and incidence of cardiovascular events that is associated with a decrease in sympathetic nervous system activity, but these drugs have such a wide array of pharmacodynamic actions that it is difficult to dissect out the therapeutic contribution of their effect on SNS [41]. In addition, they have been tested in a population of patients at cardiovascular risk not selected for diabetes or DKD [42, 43].

Our data support the concept that also in type 2 diabetes autonomic abnormalities are to some extent of functional origin, even in the presence of important complications such as renal involvement. The evident responses to oxygen are consistent with the hypothesis that tissue hypoxia, by inducing a long-term sympathetic stimulation, could play an important role in the development of autonomic dysfunction and diabetic complications.

At the same time, these findings indicate the possibility that interventions such as lifestyle and physical activity, aimed at treating multiple factors (including improvement of oxygenation), might provide more favourable results than specific interventions aimed at reducing sympathetic tone per se.

Interestingly, the increase in BRS seen during slow breathing occurred also in normoxia and was only slightly augmented by oxygen, thus suggesting that after a maximal value is reached, BRS cannot be further increased (a sort of “saturation” effect).

This finding is even more important, considering that administration of oxygen is not a physiological method to improve oxygen saturation.

Moreover, we found a small but significant interaction between group and increase in oxygen saturation in response to both slow breathing and a standard dose of 5 l/min oxygen. While this might be well explained by various confounding factors, it could be of interest to speculate that in type 2 diabetic patients an abnormality in diffusion could be partially responsible for this finding. In type 1 diabetes, it has been found that an increase in HbA1c is inversely correlated with decreased lung diffusion for oxygen [44], and previous studies in obese subjects suggest that accumulation of triglycerides could also contribute to this abnormality [45].

In our patients, both factors were significantly higher than in the healthy controls, so this possibility could not be ruled out. More studies are justified to assess this issue, as improving oxygen diffusion in the lungs (as it could be obtained by physical training) could be another method to indirectly prevent the progression of autonomic dysfunction and thus of cardiovascular and neurologic complications of diabetes.

We are aware that present study presents some limitations. It is of a relatively small size, and it could be expected that by increasing the number of observations even

stronger differences may be observed. Also we cannot exclude that the different gender distribution in the two groups and the higher BMI in the diabetic group could have influenced the differences between groups, even if the response to the dynamic tests (change in breathing pattern and effect of hyperoxia) should remain unaffected.

We did not find any relationship with years of diabetes and HbA1c levels. We believe that the small number of patients evaluated was sufficient to observe the effect of the acute interventions (as was the purpose of our study), but likely insufficient to identify more background determinants (as diabetes duration and HbA1c), for which a larger number of patients are necessary. Although we evaluated the arterial oxygenation, we could not provide information on the tissue oxygenation. Finally, since only two patients underwent renal biopsy, we considered diabetic patients with DKD of unknown origin. Thus, we cannot exclude that other underlying nephropathies may present different degree of hypoxia, autonomic dysfunction and response to breathing conditions and oxygen administration [46].

In conclusion, we believe that our data can provide a new point of view on the pathogenesis and management of diabetes complications. Further studies are needed to assess whether the improvement in BRS and the amelioration of autonomic dysfunction, obtained from breathing-induced oxygenation or by lifestyle changes like physical activity, do translate into better clinical and prognostic outcomes.

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Conflict of interest All authors declare that there is no conflict of interest.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the responsible committee on human experimentation (Ethics Committee of Foundation IRCCS Policlinico “San Matteo” of Pavia, Italy) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Human and animal rights All human rights were observed in keeping with Helsinki Declaration of 2008. There are no animal right issues as this is a clinical study.

Informed consent Informed consent was obtained from all patients for being included in the study which has been done according to the ethical standards and keeping with Helsinki Declaration of 2008.

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