BMJ Open Diabetes Research & Care

Role of kidney function on Nrf2 mRNA levels in type 2 diabetes

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To cite: Spoto B, Politi C, Postorino M, *et al*. Role of kidney function on Nrf2 mRNA levels in type 2 diabetes. *BMJ Open Diab Res Care* 2024;**12**:e003929. doi:10.1136/bmjdrc-2023-003929

Received 23 November 2023
Accepted 1 March 2024

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ABSTRACT

Introduction Diabetic kidney disease (DKD) is a major complication in patients with diabetes and the main contributor to the chronic kidney disease (CKD) global burden. Oxidative stress is a crucial factor in DKD pathogenesis but the role of the antioxidant nuclear factor erythroid 2-related factor 2 (Nrf2) and its molecular regulators has been poorly investigated in man.

Research design and methods In this case-control study, we analyzed the roles of Nrf2, a transcription factor shielding cells from oxidative stress, its repressor Kelch-like ECH-associated protein 1 (Keap1) and six microRNAs (miRNAs) that potentially suppress Nrf2. We categorized 99 participants into 3 groups: 33 non-dialysis patients with type 2 diabetes with DKD, 33 patients with type 2 diabetes without DKD and 33 control subjects and quantified the gene expression (messenger RNA (mRNA)) levels of Nrf2, Keap1 and 6 miRNAs. Moreover, we studied the correlation between gene expression levels and clinical indicators of kidney health.

Results In patients with diabetes with DKD, Nrf2 mRNA levels were significantly lower than in patients without DKD (p=0.01) and controls (p=0.02), whereas no difference in Nrf2 expression levels existed between patients without DKD and controls. Conversely, in patients with and without DKD, Keap1 expression levels were significantly higher than in controls. Of the six miRNAs studied, miRNA 30e-5p showed differential expression, being markedly reduced in patients with DKD (p=0.007). Nrf2 mRNA levels directly correlated with estimated glomerular filtration rate (eGFR) in patients with DKD (r=0.34, p=0.05) and in a formal mediation analysis the eGFR emerged as the first factor in rank for explaining the difference in Nrf2 mRNA levels between patients with and without DKD.

Conclusions The observed dysregulation in the Nrf2-Keap1 axis and the unique expression pattern of miRNA30e-5p in DKD underscore the need for more focused research in this domain that can help identify novel intervention strategies for DKD in patients with type 2 diabetes.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Oxidative stress is a factor contributing to diabetic kidney disease (DKD) but the role of the antioxidant nuclear factor erythroid-2-related factor 2 (Nrf2) and its molecular regulators is poorly studied.

WHAT THIS STUDY ADDS

⇒ This case-control study shows that the glomerular filtration rate is key to explain the difference in the expression levels of Nrf2 in patients with type 2 diabetes with and without renal complications.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The dysregulation in the expression pattern of Nrf2 and its regulators may identify novel intervention targets for DKD in patients with type 2 diabetes.

INTRODUCTION

Diabetic kidney disease (DKD) represents a severe complication arising in approximately one-third of individuals with diabetes.[1] Given the escalating global prevalence of diabetes, DKD has ascended as the primary cause of chronic kidney disease (CKD) and the most common reason for chronic kidney failure across numerous countries.[2]

Among the mechanisms contributing to DKD, oxidative stress stands out as a paramount pathogenic factor predisposing individuals to kidney damage in this condition.[3]

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a widespread protein responsible for shielding cells from oxidative damage. It acts as a transcription factor prompting the expression of genes coding for antioxidant proteins and detoxifying enzymes in the nucleus.[4] In the Nrf2 knockout mice, a downregulation in the expression of Nrf2 gene targets correlates with deteriorated renal function.[5] Conversely, compounds bolstering Nrf2 activity provide a defense against kidney damage in experimental models,[6] underscoring the pivotal role of Nrf2 depletion in the genesis of oxidative stress within CKD.

The Nrf2 function in redox equilibrium is controlled by a network of molecules, both at post-transcriptional and post-translational stages. As it occurs for a myriad of other factors,[7] microRNAs (miRNAs) may diminish Nrf2 availability by aligning with complementary sequences in Nrf2 mRNA. In addition, the

suppressor molecule Kelch-like ECH-associated protein 1 (Keap1) modulates Nrf2 activity through direct interaction.[8] Under standard conditions, Keap1 latches onto Nrf2, fostering its proteasomal degradation. However, during oxidative stress, oxidized Keap1 detaches from Nrf2 that, free to migrate into the nucleus, enhances the expression of antioxidant genes.[9]

The role of Nrf2 in the pathogenesis of DKD has been sparsely investigated in patients with type 2 diabetes[10] and, to our knowledge, the expression levels of Nrf2 have been examined in just one study in patients with DKD.[11] No observation has been until now produced on Nrf2 and its regulators, namely miRNAs that can potentially inhibit the Nrf2 signaling pathway and Keap1, in DKD. To probe into this problem, we performed a case-control study in patients with type 2 diabetes with and without DKD and in control subjects, examining the expression levels of Nrf2, Keap1 and six miRNAs that are potential suppressors of the Nrf2 signaling pathway.

METHODS

Patients and controls

This case-control study included 99 participants divided in 3 independent groups according to their phenotype: 33 non-dialysis patients with type 2 diabetes with DKD, 33 patients with type 2 diabetes without DKD and 33 control subjects.

Patients with DKD were recruited from the Unit of Nephrology Dialysis and Transplantation and patients with type 2 diabetes from the Diabetes Unit of the Reggio Calabria Hospital (Italy).

Inclusion criteria were age ranging from 18 to 70 years, history of diabetes for at least 10 years and clinical diagnosis of DKD (persistent estimated glomerular filtration rate (eGFR) ≤60 mL/min/1.73 m^2 and/or the presence of 24 hours urinary protein) or documentation of the absence of DKD (eGFR >60 mL/min/1.73 m^2 and 24 hours urinary protein <300 g/die). A control group of individuals without kidney dysfunction or damage (as defined above) and clinical signs of diabetes (defined as fasting glycemia >126 mg/dL or random glycemia >200 mg/dL, in at least two independent checks or being in antidiabetic therapy) was also enrolled into the study. This group was mainly composed by the staff members of the Nephrology Dialysis and Transplantation Unit. Patients and controls were accurately matched as for age (±2 years) and sex. All the participants were in stable clinical condition and none had intercurrent infections or acute inflammatory processes at enrollment. None were transplanted, pregnant or affected

Table 1 Demographic, clinical and biochemical data of the population grouped according to patient phenotype

	Control subjects (n=33)	Patients without DKD (n=33)	Patients with DKD (n=33)
Age, year	65±6	65±6	65±6
Male sex, n (%)	19 (57.6%)	19 (57.6%)	19 (57.6%)
Smoker, n (%)	17 (53.1%)	9 (37.5%)	17 (58.6%)
Systolic blood pressure, mm Hg	127±14	129±14	142±18**°
Diastolic blood pressure, mm Hg	73±7	71±6	71±10
BMI, kg/m^2	27±4	29±4	31±5*°
Cardiovascular comorbidities, n (%)	10 (31.3%)	6 (25.0%)	17 (54.8%)*°
Total cholesterol, mg/dL	195±44	148±33	154±43**
Glycemia, mg/dL	93±14	138±33	145±32**
C reactive protein, mg/L	1.3 (0.9–2.9)	1.2 (0.6–3.0)	3.3 (0.6–6.7)°
eGFR (mL/min/1.73 m^2)	87.5±16.7	97.4±35.9	41.2±22.7**°°
Albumin, g/dL	4.25±0.28	4.30±0.26	4.18±0.29
Creatinine, mg/dL	0.8 (0.7–1.0)	0.8 (0.7–1.0)	1.6 (1.4–2.6)**°°
Hemoglobin, g/L	143±14	133±13	120±12**°°
HbA1c, %	-	7.0±1.0	7.2±1.1
24 hours urinary protein, g/die	-	0.01 (0.00–0.10)	0.20 (0.00–0.45)°

Continuous data are expressed as mean±SD, median and IQR and among-group and between-group comparisons are made by analysis of variance, Kruskal-Wallis test, t-test and Mann-Whitney U test, as appropriate. Binary variables are summarized as absolute numbers and percentages and compared by the χ^2 test.
*p≤0.05; **p≤0.001: comparisons among groups.
°p≤0.05; °°p≤0.001: comparisons between patients with diabetes with and without DKD.

BMI, body mass index; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c.

by liver disease, diseases in the terminal phase or had a history of malignancy during the 5 years preceding the study.

Laboratory measurements

Blood sampling was performed in the early morning after an overnight fast. Serum glucose, cholesterol, triglycerides, albumin, hemoglobin, creatinine and C reactive protein were measured by standard methods in the routine clinical laboratory of our hospital. eGFR was calculated by using the 4-variable MDRD study equation.[12] Body mass index (BMI) was calculated according to the formula BMI=weight (kg)/(height (m))^2.

Gene expression analysis

Levels of mRNA and miRNA were quantified in the peripheral blood mononuclear cells (PBMCs) by using real-time PCR.

Blood samples with K_2EDTA were diluted 1:1 in phosphate-buffered saline (PBS) and cells were separated by centrifugation at 2400 rpm for 15 min in a Ficoll gradient (Lympholyte, lymphocyte isolation solution, Cedarlane, CA, USA). PBMCs were collected and washed with PBS. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, MA, USA) and miRNA by the mirVana miRNA Isolation Kit (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions. RNA and miRNA concentration and quality were evaluated by using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

Total RNA was decontaminated from genomic DNA by the DNA-free kit (Ambion, TX, USA). Single-stranded complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) and then pre-amplified with TaqManPreAmp Master Mix Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions.

For mature miRNA expression analysis, cDNA was synthesized using TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, CA,USA). Prevalidated TaqMan Gene Expression Assays (Applied Biosystems, CA, USA) were used to detect the gene expression of Nrf2 (Hs00975961_g1) and Keap1 (Hs00202227_m1) while the prevalidated Taqman Advanced miRNA Assays (Applied Biosystems, CA,USA) were used to measure the expression of miR-155-5p (483064_mir), miR-150-5p (477918_mir), miR-125b-5p (477885_mir), miR30e-5p (479235_mir), miR-28-5p (478000_mir) and miR-93-5p (478210_mir). Quantitative real-time PCR analysis was performed using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, CA,USA).

All genes and miRNA were run in duplicate and controls with no template cDNA were introduced in each plate. Target genes were considered unexpressed if the threshold cycle (Ct) value was ≥38. To correct gene expression of target genes for variation in RNA amounts and efficiency of enzymatic reactions, mRNA levels of Nrf2 and Keap1 were normalized to PGK1 (Hs99999906_m1) and GAPDH (Hs99999905_m1) gene expression while the miRNA levels were normalized to miR-191-5p (477952_mir) and miR-26a-5p (477995_mir). The identification of the reference genes for mRNAs (PGK1 and GAPDH) and miRNAs (miR-191-5p and miR-26a-5p), as the best combination for accurate normalization among a selection of eight candidate reference genes and four reference miRNA, was carried out by the statistical algorithm geNorm (software qBase, Biogazelle). The expression level of the target genes Nrf2 and Keap1 as well as of the miR-155-5p, miR-150-5p, miR-125b-5p, miR30e-5p, miR-28-5p and miR-93-5p was calculated using the comparative Ct method, expressed as $2^{-[delta][delta]Ct}$ (fold difference) and reported as arbitrary units (AU). Data analysis was performed by the software qBase.

Statistical analysis

Data are expressed as mean±SD for normally distributed data, median and IQR for non-normally distributed data and as per cent frequency. Comparisons between two groups were made by t-test, Mann-Whitney U test or χ^2 test while comparisons among more than two groups where performed by analysis of variance and Kruskal-Wallis test, as appropriate. Correlations between two continuous variables were assessed by the Pearson's product moment correlation coefficient (r) and p values. Variables having a positively skewed distribution were log transformed (Ln) before the correlation study.

The independent relationship between Nrf2 expression levels (dependent variables) and DKD versus diabetes without DKD (key independent variable) was investigated by univariate (unadjusted) and multiple linear regression analyses. In these analyses, the relationship between the clinical phenotype (DKD vs diabetes only) and Nrf2 gene expression levels was analyzed in models of increasing complexity. In particular, model 2 included the eGFR as potential mediator (ie, the key variable explaining the difference in Nrf2 gene expression levels between patients with diabetes with and without DKD) and model 3 a series of potential confounders (ie, variables which differed between patients with diabetes with and without DKD with p<0.15). The mediation analysis was performed by the model PROCESS V.3.3.[13] Data are expressed as standardized regression coefficient (β) and p value. Data analysis was performed by a standard statistical package (SPSS for Windows, V.19, Chicago, Illinois, USA).

RESULTS

Demographic, clinical and biochemical characteristics of the whole population and the three study subpopulations are reported in table 1.

According to the study protocol, the mean age (65 years) and the percentage of males (57.6%) were strictly comparable (table 1). Average systolic blood pressure, BMI and glycemia were progressively higher from controls to patients without and with DKD whereas

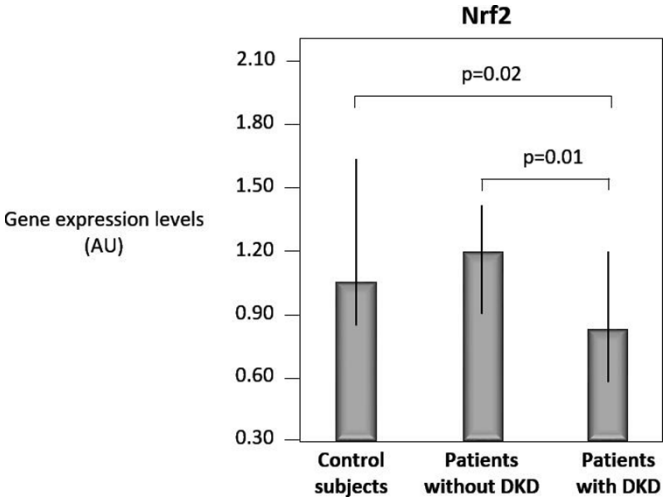

Figure 1 Nrf2 gene expression levels. Levels of Nrf2 mRNA in control subjects, in patients with type 2 diabetes without DKD and in patients with type 2 diabetes with DKD. Gene expression levels were calculated using the comparative Ct method (ΔΔCt), expressed as arbitrary units (AU) and reported as median and IQR. Pairwise comparisons were performed by Mann-Whitney U test. Ct, threshold cycle; DKD, diabetic kidney disease; mRNA, messenger RNA; Nrf2, nuclear factor erythroid 2-related factor 2.

hemoglobin had an inverse trend among the three populations (table 1). The percentage of patients with cardiovascular comorbidities was higher in the DKD group and patients in this group had also significantly higher C reactive protein and creatinine when compared with controls and patients with diabetes without DKD (table 1). As expected, the eGFR was significantly lower in patients with DKD than in the other two populations.

Gene expression levels of Nrf2, Keap1 and miRNA

In patients with DKD, Nrf2 mRNA levels were significantly lower than in patients with diabetes without DKD (p=0.01) and controls (p=0.02) whereas no difference in Nrf2 gene expression existed between patients with diabetes without DKD and controls (1.19 AU, IQR 0.90–1.38 AU vs 1.04 AU, IQR 0.87–1.66 AU) (figure 1). Conversely, Keap1 gene expression levels were almost identical in patients with diabetes with and without DKD (1.15 AU, IQR 0.63–2.14 AU vs 1.13 AU, IQR 0.79–1.73 AU) and were also significantly higher than those observed in controls (0.79 AU, IQR 0.49–1.08 AU) (p ranging from 0.01 to 0.046).

Expression levels of miRNAs were comparable in the three groups except for miR30e-5p that was significantly lower in patients with DKD than in those without (0.91 AU, IQR 0.76–1.10 vs 1.07 AU, IQR 0.92–1.25; p=0.007) (figure 2).

Correlates of Nrf2 gene expression

The expression levels of Nrf2 and miR30e-5p were unrelated in controls as well as in patients with diabetes with and without DKD (p≥0.43).

Nrf2 gene expression levels were inversely related to C reactive protein (r=−0.34, p=0.05) in controls. On the other hand, Nrf2 levels were inversely associated with creatinine (r=−0.36, p=0.04) and directly with eGFR (r=0.34, p=0.05) in patients with DKD.

Etiological models of the association of Nrf2 and Keap1 gene expression levels with renal function

To identify the risk factors which explained the difference in Nrf2 gene expression levels between patients with diabetes with and without DKD, we built up etiological models of increasing complexity (models 1–4) (table 2). In an unadjusted analysis (model 1), patients with diabetes with DKD had lower levels of Nrf2 expression levels than patients with diabetes without DKD and such a difference was statistically significant (β=−0.29, p=0.02). Importantly, when eGFR was introduced into the model (model 2), the effect of clinical phenotype (DKD vs diabetes only) on Nrf2 gene expression levels decreased by 76% (β from −0.29 to −0.07), suggesting that the reduced eGFR is key to explain the difference in Nrf2 expression levels among patients with diabetes with and without DKD. Of note, the unadjusted and adjusted standardized β coefficients (−0.29 vs −0.07) significantly differed between them, indicating that eGFR acts as a mediator on the Nrf2-diabetic phenotype (with and without DKD) link. Further data adjustment for a series of potential confounders (model 3) only slightly reduced (−17%) the strength of the association between the clinical phenotype (DKD vs diabetes only) and Nrf2 gene expression levels, confirming that eGFR is the key factor explaining the variability in Nrf2 gene expression (table 2). The Nrf2-clinical phenotype link

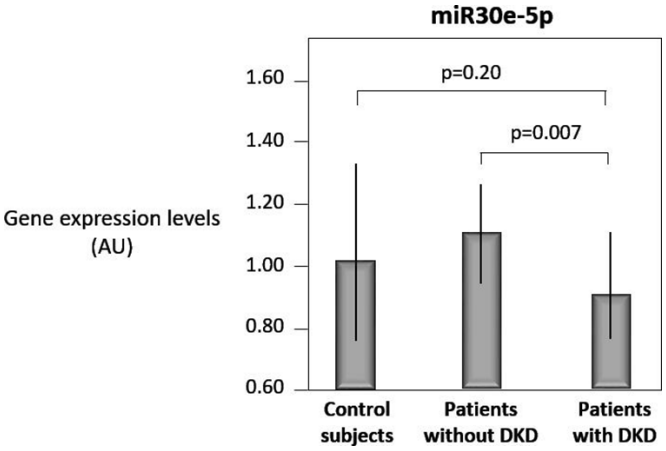

Figure 2 miR30e-5p expression levels. Expression levels of the miR30e-5p in control subjects, in patients with type 2 diabetes without DKD and in patients with type 2 diabetes with DKD. MicroRNA expression levels were calculated using the comparative Ct method (ΔΔCt), expressed as arbitrary units (AU) and reported as median and IQR. Pairwise comparisons were performed by Mann-Whitney U test. DKD, diabetic kidney disease.

Table 2 Univariate and multivariate linear regression analysis (dependent variable: LnNrf2)

	Model 1		Model 2		Model 3		Model 4	
Variables (units of increase)	Beta	p value	Beta	p value	Beta	p value	Beta	p value
Phenotype (DKD vs diabetes only)	−0.29	0.02	−0.07	0.68	−0.02	0.92	−0.02	0.93
eGFR (1 mL/min/1.73 m^2)			0.31	0.06	0.33	0.07	0.33	0.07
Systolic blood pressure (1 mm Hg)					−0.05	0.72	−0.05	0.73
BMI (1 kg/m^2)					−0.05	0.73	−0.06	0.72
Cardiovascular comorbidities (yes/no)					0.21	0.15	0.21	0.16
Smoke (yes/no)					0.06	0.67	0.06	0.70
C reactive protein (1 mg/L)					−0.06	0.70	−0.06	0.74
Hemoglobin (1 g/L)					0.02	0.88	0.02	0.88
Albumin (1 g/dL)					0.05	0.72	0.05	0.71
miR30e-5p (AU)					0.10	0.52	0.09	0.57
Keap1 (AU)							−0.02	0.86

Data are expressed as linear regression coefficients and p value.
AU, arbitrary unit; BMI, body mass index; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate.

remained identical also forcing Keap1 gene expression levels into the model (β=−0.02, p=0.93) (model 4).

DISCUSSION

This exploratory study, based on a case-control design, shows that Nrf2 gene expression, an antioxidant protective protein, is reduced in patients with DKD as compared with patients with diabetes without DKD and control subjects. Such a reduced expression was closely related to eGFR and a formal mediation analysis demonstrated that eGFR is key to explain the difference in the expression levels of Nrf2 in patients with and without DKD.

Notwithstanding its fundamental role in oxidative stress processes and its potential relevance in kidney damage in experimental models,[6] Nrf2 gene expression levels have been largely overlooked in clinical research in patients with type 2 diabetes. Just one study investigated the expression levels of Nrf2 in patients with DKD.[11] In this study, in series of 30 patients with DKD, Nrf2 levels were reduced as compared with healthy individuals and were associated with reduced levels of serum zinc. In the present study, Nrf2 levels were selectively reduced in patients with DKD but similar in patients with diabetes without DKD and in controls, suggesting that this factor impacts on kidney damage in DKD. In this regard, the direct association of Nrf2 levels with eGFR (a marker of kidney function) in DKD supports the contention that lower Nrf2 goes along with lower kidney function levels in patients with established DKD.

Keap1 is a crucial regulator of Nrf2 and an alteration in this factor may, in theory, be implicated in the reduced Nrf2 expression in DKD but, so far, no study has investigated the expression levels of this suppressor in patients with and without DKD. Diabetes per se exposes individuals to oxidative stress regardless of the presence of kidney disease. The comparable levels of Keap1 in the two diabetic groups, although higher than in controls, suggest that this factor is altered in type 2 diabetes independently of the presence of DKD. Thus, Keap1 is unlikely to be the key mediator of the oxidative stress damage that underlies DKD.

To delve into the possible mechanism(s) underlying reduced Nrf2 levels in diabetes and in DKD, we also measured six miRNAs that can potentially inhibit the Nrf2 signaling pathway. While most miRNA levels were consistent across groups, the reduced levels of miR30e-5p in patients with DKD is intriguing. miRNAs play a crucial role in gene expression regulation. If miR30e-5p is indeed involved in Nrf2 regulation, it could provide another layer to the complexity of managing oxidative stress in patients with DKD. Yet this remains a hypothetic mechanism demanding further study for implicating these miRNAs in the kidney damage resulting from the low Nrf2-related oxidative stress in DKD.

Overall, findings in this study reiterate the importance of oxidative stress in DKD. If confirmed in mechanistic studies, these data may help drive therapeutic strategies targeting the Nrf2-Keap1 pathway. Relevant miRNAs could emerge as novel treatments for DKD in patients with type 2 diabetes. Furthermore, our findings go along with clinical studies showing that Nrf2 enhancers like bardoxolone and CDDO-imidazole (1-(2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl)) may have protective effects in patients with CKD.[14 15]

This hypothesis-generating study has obvious limitations. The first limitation is the observational nature of the study that prevents causal interpretation of our results. However, strengths of our study are the fact that we took into account major potential confounders when exploring the relationship between DKD and Nrf2 gene expression levels.

In conclusion, this study has produced insights into the interplay between oxidative stress, Nrf2 and kidney function in patients with type 2 diabetes. It presents a potential pathway that could be exploited for therapeutic benefit but, as with all hypothesis-generating studies, it can only suggest further investigations to understand the therapeutic potential of interference with oxidative stress in DKD.

Contributors Conceptualization: BS; methodology: CP, RMP, AT, MP; formal analysis: GLT, BS, CP; data curation: CP, RMP; writing—original draft: BS, CZ; writing—review and editing: BS, CP, FM, CZ; project administration and funding acquisition: BS; guarantor:BS.

Funding This study was funded by Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2010/2011 n. 2010YK7Z5K-005).

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval The study protocol was approved by the Ethics Committee of the Grande Ospedale Metropolitano of Reggio Calabria (ID: 07072014_66) and all participants signed an informed consent explaining the scope and the procedures applied in the study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Not applicable.

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