NGAL and *SMAD1 gene* expression in the early detection of diabetic nephropathy by liquid biopsy

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ABSTRACT

Introduction Diabetic nephropathy (DN) is a disease that progresses with the slow and progressive decline of the glomerular filtration rate (GFR); the installation of this pathology is silent and one of the major causes of death in patients with diabetes.

Aims To identify new molecular biomarkers for early identification of the onset of DN in patients with type II diabetes *mellitus* (DM2). We studied the expression profile of the genes; *suppressor of mothers against decapentaplegic type 1* (*SMAD1*), *neutrophil gelatinase-associated lipocalin* (*NGAL*) and *type IV collagen* (*COLIV1A*) in peripheral blood and urine sediment samples.

Methods Ninety volunteers, 51 with DM2 and 39 healthy, were recruited from the Faculdade de Medicina do ABC outpatient clinic. We conducted an interview and collected anthropometric data, as well as blood and urine samples for biochemical evaluation and real-time PCR amplification of the genes of interest.

Results Gene expression data: peripheral blood *NGAL* (DM2 0.09758±0.1914 vs CTL 0.02293±0.04578), *SMAD1* (blood: DM2 0.01102±0.04059* vs CTL 0.0001317±0.0003609; urine: DM2 0.7195±2.344* vs CTL 0.09812±0.4755), there was no significant expression of COLIV1A. These genes demonstrated good sensitivity and specificity in the receiving operating characteristic curve evaluation.

Conclusion Our data suggest the potential use of *NGAL* and *SMAD1* gene expression in peripheral blood and urine samples as early biomarkers of DN.

INTRODUCTION

Type 2 diabetes mellitus (DM2) is a pathology of heterogeneous aetiology characterised mainly by hyperglycaemia, glucose intolerance, insulin resistance and hypoinsulinaemia.[1] It is a disease normally occurring in middle age (between 40 and 59 years old), its onset due to genetic factors or even due to lifestyle and inadequate lifelong diet, causing metabolic imbalances.[2 3]

Recent epidemiological data show that DM2 affects approximately 382 million individuals worldwide, representing 8.3% of the worldwide adult population. However, estimates indicate that by 2035 the projected number of diabetics worldwide is expected to grow by 366 million.[4 5] In Brazil, statistics show that there are approximately 12 million people with DM2, which generates high costs with glycaemic control and treatment of adjacent pathologies.[6 7]

This condition is directly associated with damage, dysfunction and insufficiency in several vital tissues such as kidneys, heart and blood vessels.[8] Among these dysfunctions, the one most related to the increase in mortality rates is diabetic nephropathy (DN).[9]

Clinically, DN is a syndrome that progresses with the progressive decline in the glomerular filtration rate (GFR), increased blood pressure levels (above 140/90 mm Hg), accompanied by the presence of albuminuria (>300 mg/L/24 hours) and/or increased urine creatinine (>300 mg/g/24 hours).[10 11] However, primary renal morphological and functional changes observed in DM2 may appear in normoalbuminuria and this substantially increases the risk of renal disease progression.[12]

DN is defined by characteristic structural and functional changes. The main renal functional and structural damage in DM2 is known to be related to changes in glomerular mesangial cells, characterised by thickening of the glomerular basement membrane and expansion of mesangial cells (glomerulosclerosis) and this contributes to the installation of tubulointerstitial fibrosis. Mesangial expansion occurs within the first 5 years of diabetes diagnosis and this change can be observed by microscopy.[13] Over the years, exposure to hyperglycaemia results in diffuse nodular lesions known as Kimmelstiel-Wilson lesions; however, the emergence of these lesions indicates an advanced stage of renal damage with poor prognosis.[14] All of these alterations progress towards a decrease in the number of glomeruli and the loss of nephrons, culminating in the onset of chronic renal failure.[11 14–16] Given the findings that correlate DM2 with the onset of profibrotic processes in the kidney, it is important to investigate new methods of early evaluation of DN with the purpose of preventing kidney damage and improving the quality of life of these patients.

The most commonly used method in the diagnosis of DN is microalbuminuria screening, which is one of the first indicators of the presence of incipient nephropathy in diabetics. However, this assessment is observed when kidney damage has already taken place.[15] Thus, there is a need to study molecular markers so that the early stages of DN installation are identified and preventive measures can be taken in time to avoid total impairment of renal function in patients with DM2.

One of the major components of expansion of mesangial matrix in DN is type IV collagen (COLIV). This protein is part of the structural base

of basement membranes. In the kidney, it is synthesised mainly by epithelial cells, mensangial cells and glomerulus podocytes. Physiologically, COLIV contributes to the formation of the glomerular filtration barrier that is essential for the glomerular permselectivity to proteins.[16] On the other hand, when renal podocytes are exposed to a thick COLIV network, their dysfunction is associated with the onset of glomerular disease.[17] Increased COLIV expression in renal glomeruli or tubules has been shown to mark an advanced stage of renal function loss in DM2.[18] The major transcriptional regulatory protein of COLIV is the suppressor of mothers against decapentaplegic type 1 (SMAD1).[19] SMADs are intracellular proteins capable of regulating transcription factors and expression of target genes; once activated, these proteins migrate to the nucleus, where they activate or suppress the transcription of different target genes.[20] Recent studies have found that SMADs can be detected in the urine of patients with DN, allowing the determination of the initial onset of fibrotic processes in the kidney of patients with diabetes.[21–23]

In addition to the glomerular damage already mentioned, hyperglycaemia compromises the perfect functioning of the renal tubules, triggering diabetic tubulopathy.[24] These tubular damages are part of the pathogenesis of DN and, as well as glomerular changes, produce substrates resulting from these changes, one of which is neutrophil gelatinase-associated lipocalin (NGAL). NGAL is a low-molecular weight protein (25 kDa) that belongs to the family of lipocalin proteins released from neutrophils and other epithelial cell types, including renal tubule cells. These proteins are synthesised in response to tubular lesions and have already been detected in the urine of patients with diabetes, thus being a complementary indication in the early detection of DN.[25 26] Some studies have correlated the presence of urine NGAL with time of DM2 onset, as well as blood glucose levels, suggesting NGAL is a potential non-invasive marker of the evolution of DN. The advantage of this biomarker is that it has already been detected in patients with or without albuminuria, being therefore highly sensitive for the early evaluation of tubular damage.[27] All this evidence indicates the potential importance of detecting urine NGAL for the identification of DN onset, especially the evaluation of gene expression in this substrate, that is, even before the presence of protein expression.

In light of the above, the objective of this study was to evaluate early molecular changes to subsequently support measures to prevent end-stage renal failure in DM2. Therefore, it is important to investigate the expression profile of the aforementioned genes in phases preceding DN, as well as their correlation with altered biochemical parameters in DM2.

MATERIALS AND METHODS

Design

This was a cross-sectional study . The patients were recruited at the Specialist Outpatient Clinic of *Faculdade de Medicina do ABC*.

Patients who agreed to participate in the study were given a free and informed consent form with the appropriate explanations of the adopted protocols and the informed consent forms were signed by the participants. An interview was conducted to collect participants personal data, as well as to perform anthropometric assessments (age, gender, ethnicity, height, body weight, body mass index (BMI)) and to verify the medications used for the treatment of DM2 and its comorbidities.

Participants

The control group (CTL) consisted of healthy, non-diabetic subjects with no history of prior kidney disease, liver disease or carcinoma prior to the study. All were aged 21 years or older, were not illicit drug users and had no comorbidities.

The DM2 group consisted of patients diagnosed with DM2 (fasting glucose ≥140 mg/dL and HbA1c ≥7%) for at least 5 years without impaired renal function, assessed by GFR, aged ≥21 years and under pharmacological treatment for DM2. The diagnostic criteria for DM comply with those defined by *ADA*.[3]

Ineligible volunteers were considered to be those who were undergoing dialysis treatment, had been hospitalised for any reason within 30 days prior to the consultation, patients under treatment for neoplasia, patients with AIDS, users of immunosuppressive medication, patients with liver or kidney disease or those who had undergone kidney or pancreatic transplantation.

Sample collection

The samples were collected according to the Standard Operating Protocol adopted by the Clinical Analysis Laboratory of the Centro Universitário Saúde ABC/Faculdade de Medicina do ABC. All samples were kept properly refrigerated until the time of analysis. Blood samples for determination of biochemical parameters were obtained by vacuum venipuncture after an 8-hour fast.

Evaluation of glycaemic levels in patients with DM2

Determination of fasting plasma glucose was performed by the evaluation of blood glucose concentration after night fasting, by the automated enzymatic-colorimetric method using fluorinated plasma. For this parameter, values≥140 mg/dL were considered altered.

Evaluation of glycated haemoglobin (HbA1c) levels in patients with DM2 by low-pressure liquid chromatography (LPLC)

HbA1c was determined using the LPLC technique using a DiaStat–Bio-Rad analyzer, which expresses the percentage of total haemoglobin and reflects the glycaemic status between the preceding 8–12 weeks. The collected material was 5 mL of total blood with 1 mL of haemolysed reagent. Values above 7% were considered altered.

Evaluation of kidney function in patients with DM2

To assess the kidney function of patients, serum creatinine was measured by the ELISA method. We followed the standard methodology of the Clinical Analysis Laboratory of the Faculdade de Medicina do ABC. After dosing, the estimated GFR was calculated by the Modification of Diet in Renal Disease formula.[28]

Serum creatinine dosage

Biochemical analyses of serum creatinine were determined in duplicates by the modified kinetic-colorimetric Jaffé method.

Microalbuminuria determination

The determination of microalbuminuria in isolated urine samples was performed by the Biosystems immunoturbidimetry method (BioSystems S.A. Costa Brava, Barcelona, Spain). The adopted

reference value was up to 15 mg/L for normoalbuminuric and between 30 and 300 mg/24 hours for microalbuminuric.

NGAL, *SMAD1* and *COLIV1A* gene expression in peripheral blood and in urine

Total RNA extraction

Total RNA was isolated from peripheral blood using the TRIzol method (TRIzol LS Reagent, Thermo Fisher cat. no. 10 296-010) according to the manufacturer's protocol. For total urine RNA extraction, samples (15 mL) were initially centrifuged at 4°C at 2500 revolutions/min for 10 min to obtain urine sediment. The supernatant was discarded and 1 mL TRIzol was added to the cell pellet. The extraction process followed the standard protocol for TRIzol. Total RNA concentration was estimated by spectrophotometric reading on NanoView Plus equipment (GE Health Care; NANODROP).

cDNA synthesis

Total RNA samples (initial 1 µg) obtained from peripheral blood and from urine were converted to cDNA with the aid of SSIII First Strand qPCR Supermix (Invitrogen, cat no 11752050) according to the manufacturer's protocol.

Amplification

Specific primers for the target genes were designed using Primer3 Input V.0.4.0 software (available at http://frodo.wi.mit.edu/primer3/). Primer sequences and their amplicons were: *NGAL* forward (caatgtcacctccgtcctgt) and reverse (tgctggttgtagttggtgct) (162 bp amplicon); *SMAD1* forward (ggaaacaggcgatgaagaag) and reverse (catccagagagcggggaatg) (159 bp amplicon); *COLIV1A1A* forward (ccaagggagaaaagggccaa) and reverse (cggcctatgagtcctgggta; 178 bp amplicon) for expansion of mesangial matrix evaluation. For normalisation of the relative expression of target genes, expression values of the ribosomal gene *RPL13A* (18S) forward (ttgaggacctctgtgtatttgtcaa) and reverse (cctggaggagaagagaaagaga; 126 bp amplicon) were used.

Initial standardisation of real-time PCR amplifications occurred in an Applied Biosystems 7500 Real Time PCR Systems thermal cycler (Applied Biosystems, Foster City, California, USA) in a final volume of 15 µL containing: 1X SYBR Green mix (Quantitec SYBR Green PCR QIAGEN Cat. no. 204054), 10 pmol of each specific primer and 2 µL cDNA (initially diluted 10X). The initial cyclic parameters were an initial hot start step at 95°C for 10 min, followed by 40 repetitions of 95°C for 15 s and 60°C for 25 s.

The difference in intergroup and intragroup relative gene expression was assessed by the $2^{-\Delta Cq}$. The results were presented as difference of expression followed by range (minimum and maximum).[29 30]

Statistical analysis

Results were expressed as mean±SD. These were compared using unpaired Student's t-test for parametric values and Mann-Whitney test for non-parametric values. For the correlation analysis between the studied genes and the biochemical parameters, the Spearman correlation test was performed. These analyses were performed using the computer program GraphPad Prism (GraphPad, V.6.0, USA). The significance level established was 5% (descriptive value of $p<0.05$). A receiving operating characteristic (ROC) curve analysis was performed to evaluate the accuracy and establish a cut-off point for the markers.

Table 1 Demographic data of healthy donors (control group)

Variables	Values
Gender (M/F) %	
Male %	46
Female %	54
Age (years) mean±SD	47±13
Ethnicity	
Non-Caucasians %	24
Caucasians %	76
Weight (kg) mean±SD	73±17
Height (m) mean±SD	166±10
BMI (calculation) mean±SD	26±4
Other evaluated parameters Hypertensives %	
Yes	2
No	0
Do not know	0

BMI, body mass index.

RESULTS

A total of 110 volunteers were recruited, of which 64 had DM2 and 39 were healthy (considered control, CTL). Of the 64 individuals in the DM2 group, 13 were excluded due to a prior history of liver disease, kidney disease and myasthenia gravis. Thus, a total of 90 individuals were considered viable to participate in the study.

The DM2 group consisted of 51 patients, being (66%) female and (34%) male. The mean age of the individuals in this group was 64±9 years. The CTL group consisted of 39 individuals (54%) female and (46%) male, with a mean age of 47±13 years. Other demographic data that characterise the groups in this study are described and discriminated in tables 1 and 2.

When assessing the duration of diabetes of patients in the DM2 group, we found that 72% of the individuals were diagnosed with DM2 for less than 5 years, 10% of the individuals for less than 10 years and 18% of the patients had DM2 for more than 10 years. When comparing the DM2 group to the CTL group, we identified a statistically significant difference for BMI

Table 2 Demographic data of participants with type II diabetes *mellitus*

Variables	Values
Gender (M/F) %	
Male %	34
Female %	66
Age (years) mean±SD	64±9*
Ethnicity	
Non-Caucasians %	26
Caucasians %	69
Weight (kg) mean±SD	77±16
Height (m) mean±SD	162±11
BMI (kg/cm^2) mean±SD	29±5*
Other evaluated parameters Hypertensives %	
Yes	61
No	28
Do not know	11

*P<0.05 versus control group Student's t-test.
BMI, body mass index.

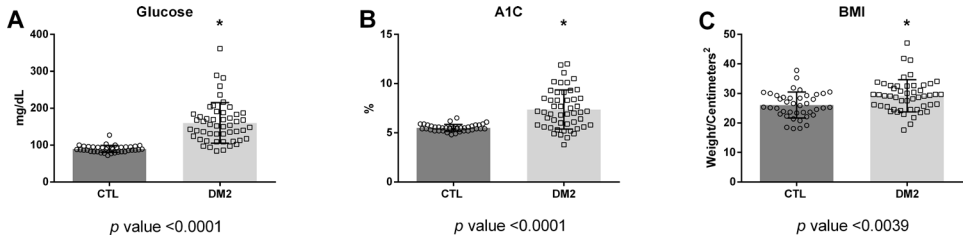

Figure 1 Graphical representation of (A) fasting glucose, (B) glycated haemoglobin (A1c), and (C) body mass index (BMI) data from participants with type II diabetes mellitus (DM2) compared to healthy participants. Values expressed as mean±SD. Student's t-test, *p <0.05 versus control group (CTL).

values (DM2 29.21±5.5, *p<0.05; CTL 26.06±4.4 kg/cm^2). Sixty-one per cent of the diabetic individuals had arterial hypertension (AH) compared with 2% of healthy individuals (tables 1 and 2).

The DM2 group presented fasting glucose and A1C values compatible with the expected values for this study, as shown in figure 1. These group characterisation data are within the DM2 diagnostic reference values established by The American Diabetes Association 2018–2019 (fasting blood glucose ≥126 mg/dL and A1C≥6.5%) (3).

From data exclusively reflecting possible renal impairment, we found increased values for plasma urea (DM2 36.1±10.3* vs CTL 29.7±6.5 mg/dL) and microalbuminuria (DM2 50±81* vs 10±13 mg/L) in patients with DM2 compared with healthy individuals. We categorised participants with DM2 according to mean urinary albumin excretion (US) as: normoalbuminuric (US≤26 mg/g creatinine), microalbuminuric (US>26 mg/g) and macroalbuminuric (≥300 mg/g). We did not find a correlation between microalbuminuria values and blood glucose levels (r=−0.2955, p=0.1345).

There was no change in plasma creatinine (DM2 0.76±0.2 vs CTL 0.73 0.2 mg/dL), GFR (DM2 100±18 vs CTL 108±25 mL/min/1.73 m^2) and proteinuria (DM2 19±41* vs CTL 9±5 mg/dL) in participants with DM2 compared with healthy donors (figure 2).

Peripheral blood *NGAL* gene expression was increased in patients with DM2 compared with healthy individuals (DM2 0.09758±0.1914* $2^{-\Delta Cq}$ vs CTL 0.02293±0.04578). In the urine sediment, however, we did not observe a significant alteration of urinary *NGAL* expression (DM2 0.1219±0.6798 $2^{-\Delta Cq}$ vs CTL 0.1130±0.2799; figure 3).

In our evaluation of *SMAD1* expression, we found a prominent increase in the expression of this gene in patients with diabetes in both peripheral blood and urine sediment samples (blood: DM2 0.01102±0.04059* $2^{-\Delta Cq}$ vs CTL 0.0001317±0.0003609; urine: DM2 0.7195±2.344* $2^{-\Delta Cq}$ vs CTL 0.09812±0.4755; figure 3).

In the evaluation of *COLIV1A* gene expression, we identified that only four participants with diabetes expressed this gene in peripheral blood and three expressed this gene in urine sediment. Among the healthy participants, none expressed *COLIV1A* in either peripheral blood or urine sediment. In these patients, we found no correlation between the expression of this gene and biochemical or anthropometric data.

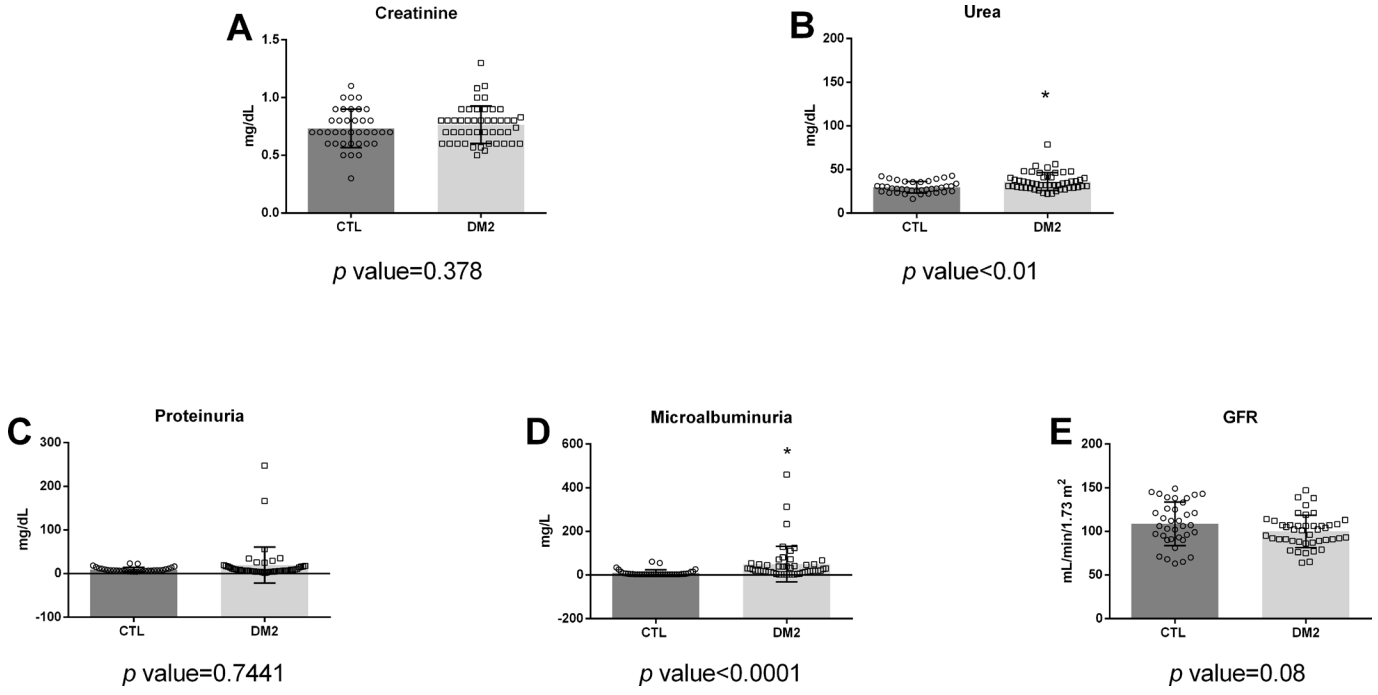

Figure 2 Graphical representation of (A) plasma creatinine (mg/dL), (B) urea (ng/dL), (C) proteinuria (mg/dL), (D) microalbuminuria (mg/L) and (E) glomerular filtration rate (GFR; mL/min) 1.73 m^3 of participants with type II diabetes mellitus (DM2) compared to healthy participants. Values expressed as mean±SD. Student's t-test and Mann-Whitney test, *p <0.05 versus control group (CTL).

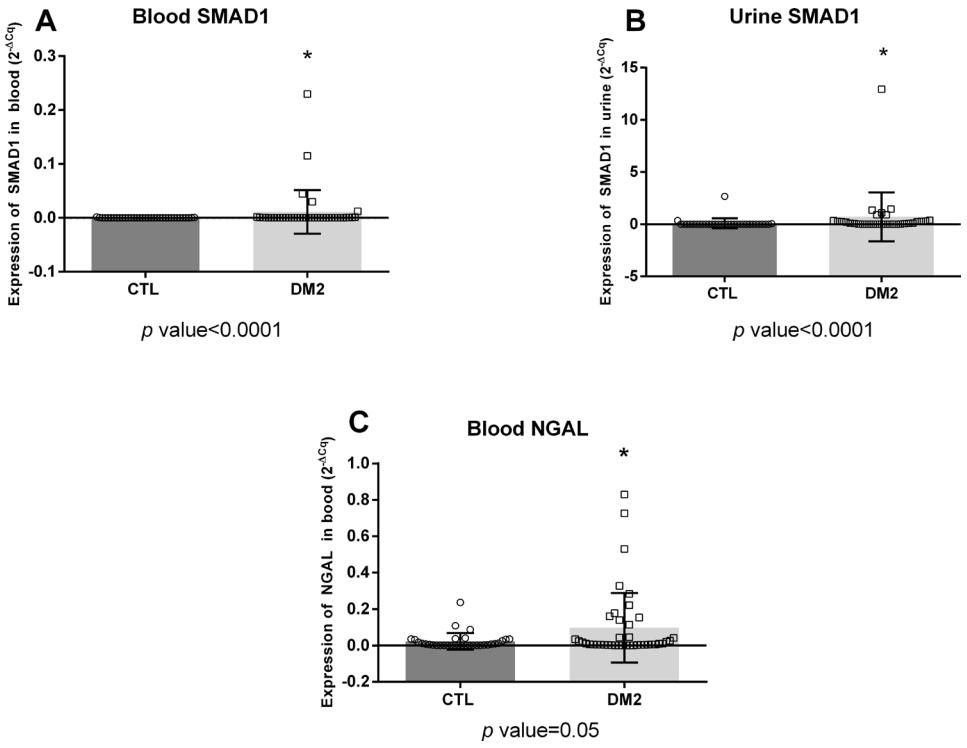

Figure 3 Graphical representation of suppressor gene expression data from suppressor of mothers against decapentaplegic type 1 (SMAD1) in peripheral blood (A) and urine sediment (B) samples; expression of the neutrophil gelatinase-associated lipocalin (NGAL) gene in peripheral blood (C) of participants with type II diabetes mellitus (DM2) compared to healthy participants (CTL) $2^{-\Delta Cq}$. Values expressed as mean±SD. Mann-Whitney, *p <0.05 versus control group (CTL).

Finally, when performing ROC curves to identify the specificity and sensitivity of the *SMAD1* and *NGAL* genes, we obtained the following indexes: *SMAD1* blood (cut-off >3.06e−005, 90% sensitivity, 77% specificity, area 08457, SE 0.04963, 95% CI 0.7484 to 0.9430, p<0.0001); urine *SMAD1* (cut-off >10.13, 63% sensitivity, 94% specificity, area 0.8948, SE 0.04291, 95% CI 0.8107 to 0.9789, p<0.0001); blood *NGAL* (cut-off >0.1109, 27% sensitivity, 97% specificity, area 0.6327, SE 0.06482, 95% CI 0.5056 to 0.7597, p=0.05). ROC curve values for urine *NGAL* were not statistically significant (figure 4). We also evaluated whether there would be a correlation between SMAD1 expression in urine and blood samples in patients with DM2, and likewise investigated NGAL expression in both liquid matrices. We found no correlation of NGAL expression between the two matrices (r^2=0.07147, p=0.674); on the other hand, SMAD1 gene expression in urine correlated positively with the expression of the same gene in blood (r^2=0.4163, p=0.024).

We did not identify a relationship between the expression values of the studied genes with the other data while evaluating if there would be correlation between them. We only found a correlation between A1C values and *SMAD1* expression in the urine of patients with DM2 (r^2 0.3734, p=0.05), as shown in figure 5.

DISCUSSION

The present study performed a molecular exploration, specifically regarding the expression of genes in peripheral blood and urine samples related to the onset of DN, what can be called a liquid biopsy.

We studied the expression profile of genes involved in the installation of DN, as well as the evaluation of the biochemical and anthropometric state of diabetics in order to obtain new molecular biomarkers to aid in the early diagnosis of DN.

The main available disease management measure for the prevention of DN onset is glycaemic and HbA1c control; however, the period of safe exposure to high glycaemic levels has not been established, nor have the levels responsible for initiating tubular or glomerular alterations.[31–33] In our data, we observed that, regardless of the time since diagnosis of DM2, changes in some of the markers related to renal injury, such as *NGAL* and *SMAD1*, are already present.

The most surprising finding in this study was increased *SMAD1* and *NGAL* gene expressions in patients with diabetes without altered renal function or GFR. For this evaluation, we used peripheral blood and urine samples, that is, simple and non-invasive collection samples. With this, we identified two early molecular markers with high accuracy for DN installation to be used in the diagnosis of this disease.

In recent years, urine has been considered the most promising biological fluid when it comes to identifying functional alterations in the kidneys.[34] Our study demonstrates the possibility of evaluating the expression of genes related to early tubular and glomerular changes in the urine sediment of patients with GFR within normal ranges.

SMAD1 expression was increased in both urine and peripheral blood samples of patients with DM2. SMAD1 is well established as a protein present in diabetic glomerulopathy. Doi *et al* (2018) investigated the presence of this protein in the urine of patients with DM2 and reported that SMAD1 is directly associated with mesangial matrix expansion, a classic feature of DN. In addition, increased urinary levels of SMAD1 have been found to precede the decline of GFR and of macroalbuminuria.[35] SMAD1 protein

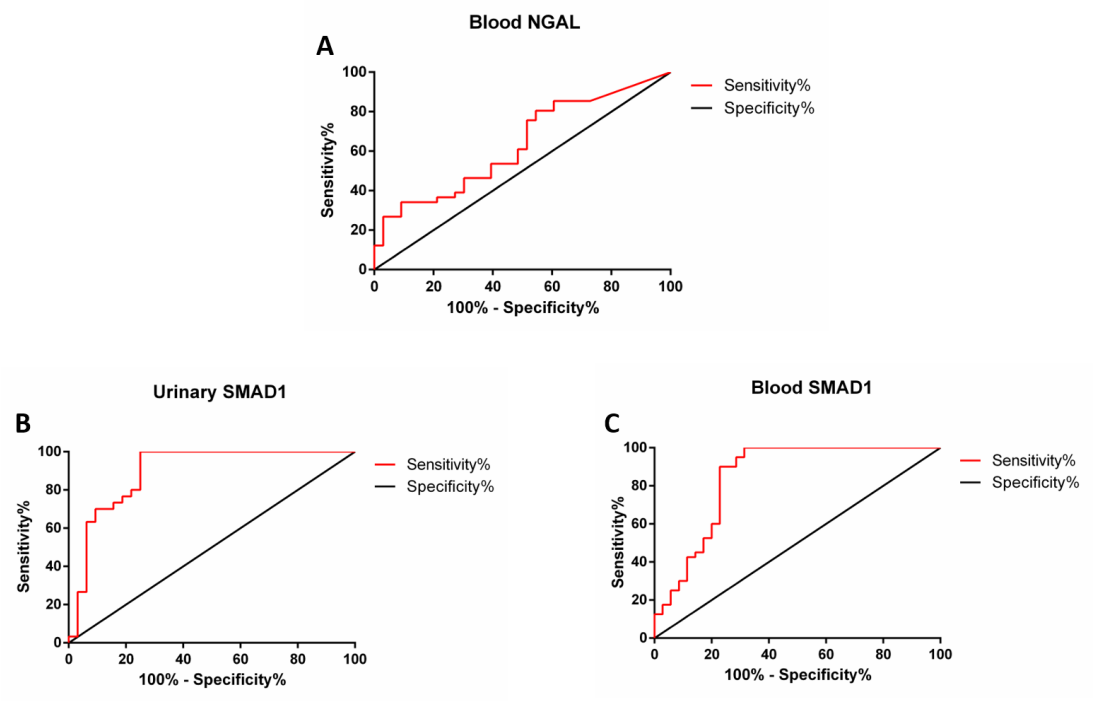

Figure 4 Graphical representation of receiver operating characteristic curve data from gene expression; suppressor of mothers against decapentaplegic type 1 (SMAD1) in peripheral blood samples (A); and in urine sediment (B); and the neutrophil gelatinase-associated lipocalin (NGAL) gene in peripheral blood (C) of participants with type II diabetes mellitus ($2^{-\Delta Cq}$).

expression had already been identified as a marker of mesangial matrix expansion in rats in the early phase of DN.[36] However, to our knowledge, there are no studies that have shown gene expression of this molecule in two distinct liquid biological matrices, so our study is apparently the first to suggest the use of SMAD1 gene expression in the early diagnosis of DN in liquid samples. In addition, the expression of this gene was positively correlated in the two liquid matrices studied, showing once again the possibility of using this marker as an indication of early renal damage in diabetics.

Chen *et al* (2019) studied the signalling pathway of bone morphogenetic protein 4—BMP4/SMAD1 in vitro and showed that exposure to high glucose concentrations activates the SMAD1 pathway inducing COLIV protein expression, the main marker for renal fibrosis.

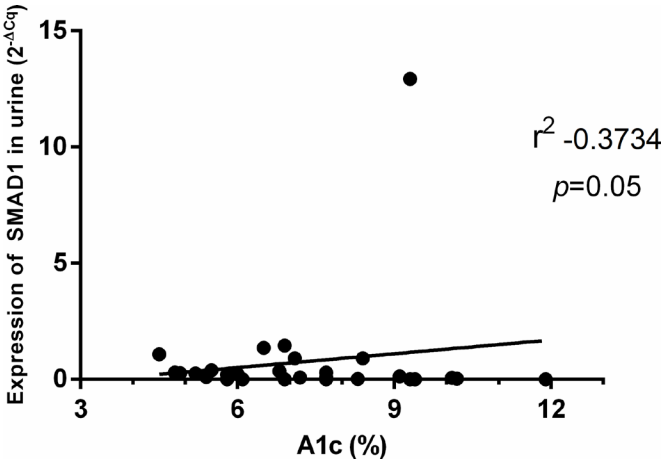

Figure 5 Graphical representation of the correlation between suppressor of mothers against decapentaplegic type 1 (SMAD1) gene expression in the urine of patients with type II diabetes mellitus and blood glycated haemoglobin (A1c) values. Spearman correlation test.

On the other hand, blocking this pathway inhibits the synthesis and deposition of COLIV, definitively proving the participation of SMAD1 in the activation of COLIV.[37] Since COLIV is the last step in the activation pathway of renal tissue fibrosis, and in this study, we were unable to identify changes in the expression of this gene, we can assume that our patients are at a very early stage of renal alterations, a state which has not yet been identified by the most commonly used biochemical markers.

Taking into consideration the importance of the SMAD1 signalling pathway in the mesangial alterations present in DN, our study suggests that a higher expression of the *SMAD1* gene in incipient DM2 may signal the onset of this nephropathy. To date, this relationship has not been determined in patients with diabetes without renal dysfunction. In addition, we found a strong correlation trend between the expression of urine *SMAD1* and A1c, which leads us to believe the DM2-induced metabolic imbalance directly interferes with the increase in SMAD1. This relationship had already been proposed in another study in which the authors evaluated the relationship between urine SMAD1 protein expression and A1c values; however, this had not yet been verified in relation to *SMAD1* gene expression. The main hypotheses credited to this effect are: alteration in renal haemodynamics, increased activity of the p38 MAP kinase pathway and accumulation of ransforming growth factor (TGF)-β1.[38]

We verified a different expression profile for *COLIV1A* in our diabetics, since of the 53 patients evaluated only 4 presented the expression of this gene in the blood and 3 in the urine sediment. These data justify the absence of *COLIV1A* expression in most diabetics, since SMAD1 is a precursor in the signalling cascade for expression of this molecule. In healthy participants, there was no expression of *COLIV1A*, evidence that this molecule is the last to be altered and when it is present the loss of renal

function is evident. The SMADs act as TGF-β adjuvants in the transcription of 1α and 2 COLIV chains, and this is the main component of the mesangial matrix expansion.[39] Among the four patients with DM2 who expressed *COLIV1A*, we found no correlation between the expression of this gene and any of the other studied parameters.

Our results suggest alterations in *SMAD1* gene expression without reduction in GFR, data confirmed by Doi *et al* (2018), who speculated that the presence of SMAD1 in urine precedes the decline of GFR in diabetics.[35]

When investigating the expression of the *NGAL* gene, we found increased expression only in blood samples. One study showed the relationship between the urinary excretion of NGAL and that of matrix metalloproteinase 9 (MMP-9), an important gelatinase in the degradation and renewal of proteins that make up the extracellular matrix. In that study, the authors emphasise that NGAL inhibits the degradation of MMP-9, which is found in renal podocytes when there is vascular impairment.[40] Thus, we can suggest that increased expression of systemic *NGAL* is contributing to the increased proteolytic activity of MMP-9; with time, this increase in proteolytic activity will cause the degradation of renal structural proteins and only then will we be able to verify possible changes in the expression of this gene in urine. NGAL is known to be a molecule released by neutrophils in acute inflammatory processes and in the innate immune response to various pathophysiological conditions.[41] In this study, we did not evaluate other markers of inflammation as they were not the focus of our investigation. However, we know that DM2 is an inflammatory metabolic disease and we believe that increased NGAL expression in the blood samples collected from our patients is especially mediated by the inflammatory process characteristic of DM2. Evaluation of the expression of this gene in two different matrices helped us discriminate systemic expression from expression in renal epithelial cells. It is much more effective as a marker of inflammatory process at this stage of DM2 than specifically for early renal changes, at least when it comes to gene expression.

It is possible in DM2 differentiated systemic expression will occur, regardless of alterations in expression in renal tissue or urine sediment. The mechanisms responsible for this change in expression occurring primarily in the blood are not fully described in the literature; apparently, urine sediment findings do not reflect changes in systemic expression. However, further evaluations should be made to confirm this hypothesis.

To confirm the accuracy of these genes as possible biomarkers of nephropathy, we performed an ROC curve analysis. ROC curve analysis is used to establish marker cut-off values for diagnosis of the disease, indicating the sensitivity (ability to detect disease) and specificity values (ability to minimise false-positive results) for each value obtained between the individuals of the study. The area under the curve ranges from 0 to 1 and is related to the accuracy of the test in diagnosing the disease.[42] In this study, we obtained high values of sensitivity and specificity for the studied genes, inferring that these biomarkers may be used in diagnostic practice. Our sensitivity and specificity values for *SMAD1* and *NGAL* gene expression were higher than those found for a classic marker such as microalbuminuria.[43] However, we know that evaluations with a larger sample size will be necessary before the implementation of these markers in the diagnostic routine.

In this study, participants with DM2 had higher BMI values than CTL and were considered obese. The relationship among obesity, DM2 and kidney disease is well established.[44–47] Higher adiposity favours an increase in the leptin hormone and other adipokines, as well as a decrease in adiponectin. Consequently, there is increased insulin resistance, hyperinsulinaemia and activation of the renin angiotensin aldosterone system. This activation cascade favours the potentiation of inflammatory processes and the increase of reactive oxygen species. All of these changes are highly deleterious to structural cells of the kidneys, gradually decreasing renal function.[47 48] In light of this, BMI is suggested as a potential risk factor for kidney disease in patients with diabetes, and our data confirmed this hypothesis.

In addition to the deleterious effect of obesity on renal function, AH is also present in our participants with DM2. There are studies that widely discuss the direct influence of increased blood pressure levels on glomerular damage. AH strongly influences the decline of renal function, and one of the main mechanisms involved is the hyperactivation of the renin angiotensin aldosterone system, which promotes volume overload and potentiates proinflammatory, prooxidant and profibrotic mechanisms.[48] AH promotes increased glomerular hydrostatic pressure, transcapillary flow and macromolecule ultrafiltrate, all of which are responsible for irreparable damage to glomeruli.[49] The presence of AH and increased BMI in our patients with DM2 justify the alterations in some of the parameters related to renal dysfunction in this study, especially the increase in urea and microalbuminuria.[50]

Among the biochemical markers, microalbuminuria is considered to be the measurement of choice for the detection of early kidney disease.[51–53] Despite being part of renal evaluation screening in clinical practice for many years, it should be observed in conjunction with GFR (formula that encompasses age, gender, ethnicity and body surface). Microalbuminuria is also elevated in states of generalised vascular damage and has been validated as an established marker for cardiovascular prognosis.[51] Hyperglycaemia is known to not be the only factor involved in this alteration. Factors such as AH, gender, age, race, smoking and obesity may also increase microalbuminuria levels, so this marker cannot be considered to be unique for tubular or glomerular damage[54–56] and when detected there is already kidney damage. A study with rats showed that SMAD1 is a better diagnostic marker than albuminuria in evaluating the expansion of the mesangial matrix present in DN.[57]

The relationship between the main biochemical markers of renal injury with GFR has been studied in type 2 diabetics. According to the authors, patients with reduced GFR but no change in proteinuria or microalbuminuria had a lower risk of end-stage renal disease than other participants in the study.[58] However, it is known that there are non-albuminuric nephropathic patients and, for this reason, studies of gene expressions of more specific molecular markers have grown in recent years.[59]

Take home messages

- Neutrophil gelatinase-associated lipocalin (NGAL), suppressor of mothers against decapentaplegic type 1 (SMAD1) and type IV collagen (COLIV) protein expressions have been studied as biomarkers for kidney diseases. The measurement of these molecules is already applied in clinical practice in biopsies, blood and urine samples.
- The present study shows the possibility of early detection of NGAL and SMAD1 gene expression in liquid biopsy (blood and urine samples) of patients with diabetes, for early detection of diabetic nephropathy, before the significant structural changes.
- This preliminary study brings advances in early diagnostic for diabetic nephropathy, contributing to prophylactic measures in these patients.

In this study, two molecular biomarkers of renal damage that were altered even before changes in classic biochemical markers or GFR could be identified. This preliminary study showed the possibility of using liquid samples (blood and urine) as a diagnostic tool for DN, as well as proving the possibility of evaluating the gene expression of markers of kidney disease in a non-invasive manner. We are aware that this study should be expanded before these markers are used in clinical practice, but we point to a breakthrough in the area of liquid biopsy in a nephrological disease.

CONCLUSION

Our data suggest the potential use of *NGAL* and *SMAD1* gene expression in liquid biopsy samples such as urine and blood. We have shown that even without changes in GFR or other markers of renal function, the expression of these two genes is increased; therefore, they can be considered as early biomarkers of DN.

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