Title: A cross-sectional study of *p66Shc* Gene Expression in Liquid Biopsy of Diabetic Patients. Is it possible to predict the onset of renal disease?

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Authors Contribution Statement:

Contributor Role	Role Definition	Authors 1	2	3	4	5	6	7	8	9	10	11	12
Conceptualization	Ideas; formulation or evolution of overarching research goals and aims.	X										X	X
Data Curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse.		X	X								X	X
Formal Analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data.												X
Funding Acquisition	Acquisition of the financial support for the project leading to this publication.	X										X	X
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.	X	X	X	X	X	X	X	X	X	X		
Methodology	Development or design of methodology; creation of models		X	X	X	X	X	X					
Project Administration	Management and coordination responsibility for the research activity planning and execution.	X										X	X
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools.			X								X	X

Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components.			X									X
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.											X	X
Validation	Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs.		X	X								X	X
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.	X	X	X	X	X	X	X	X	X	X	X	X
Writing – Original Draft Preparation	Creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation).	X										X	X
Writing – Review & Editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or post-publication stages.	X		X								X	X

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Discussion Points:

1. *p66Shc* gene expression in liquid biopsy does not predict renal disease installation in diabetic patients.
2. There was no association between *p66Shc* gene expression and the other laboratory variables that were studied.
3. Minor changes in *p66Shc* gene expression may signal the dysregulation of the oxidative system.

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ABSTRACT.

Background: Diabetic nephropathy (DN) is a disorder affecting glomerular function that, histologically, is due to the presence of glomerulosclerosis accompanied by endothelial dysfunction of the afferent and efferent renal arterioles. Insulin resistance in diabetic patients is known to be one of the causes of endothelial dysfunction because it increases oxidative stress, and one of the main genes regulating the production pathways of reactive oxygen species is *p66Shc.* The aim of this study was to evaluate the *p66Shc* gene expression as a precocious biomarker of renal dysfunction in diabetes patients, using liquids samples urine sediment and peripheral blood.
Methods: 29 diabetic patients and 37 healthy donors were recruited from the Centro Universitário FMABC outpatient clinic. It was evaluated *p66Shc* gene expression by RT-qPCR technique in urine and peripheral blood samples from diabetic patients which were compared with healthy donors.
Results: There was no significant expression of *p66Shc* gene in samples from diabetic patients compared with healthy donors. However, *p66Shc* expression in blood of diabetics was 3.6 times higher in diabetics ($0.02417\pm0.07865^{2-\Delta CT}$, n=29) than in healthy participants (0.00689±0.01758, n=37) and in urine, it was 1.48 times higher in diabetics group ($0.02761\pm0.0541^{2-\Delta CT}$) than in CTL group (0.0186±0.02199).
Conclusion: There was no significant *p66Shc* gene expression in peripheral blood and urine samples of diabetic patients without kidney injury in comparison with health donors, although there is a tendency for this gene to participate in the oxidative imbalance present in diabetes.

Keywords: Diabetes Mellitus, *p66Shc*; Biomarker; Liquid biopsy.

INTRODUCTION.

Diabetes Mellitus is a heterogeneous disorder defined by the presence of hyperglycemia due to the functional insufficiency of insulin action on its receptor.[1, 2] Currently, there is improved survival of diabetic patients and, in parallel, increased chances of developing chronic complications due to the period of exposure to hyperglycemia, among these complications is nephropathy, the main reason for admission of patients to dialysis and transplantation programs.[3, 4]

Diabetic nephropathy (DN) consists of a disorder that affects glomerular function, histologically, occurs due to the presence of glomerulosclerosis, a condition in which the basal membranes of the glomerular capillaries are thick and the mesangium, which surrounds the glomerular vessels, is increased due to deposition of extra cellular matrix (ECM).[5, 6] This is an asymptomatic disease that is rarely identified in the early stages and is therefore considered potentially serious. It is detected between the moderate and late phases. This disease usually presents three clinical phases that allow classification of patients according to its progression.[7, 8]

Endothelial nitric oxide synthase (eNOS) synthesis is impaired in patients with T2DM, due to factors such as hyperglycemia, hyperinsulinemia and insulin resistance, which leads to one of the main factors involved in the physiopathology of DN: a dysfunction of endothelial glomerular capillaries and afferent and efferent arterioles due to increased production of ROS and reduced production of nitric oxide (NO). This condition results in vasoconstriction and endothelial oxidative stress that causes significant cell death and worsening of the condition of the patient with DN.[9, 10]

Modulating the oxidative stress process is the p66Shc protein, which is an isoform of the *SHC1* gene, located in the first chromosome. p66Shc acts on the endothelial cell by increasing the production of ROS through three different mechanisms, in the cell nucleus in the cell membrane and in the mitochondria, In the nucleus, p66Shc is mediated by Forkhead Box Sub-Group O (FOXO), resulting in decreased expression of the enzymes ROS-scavenging catalase (CAT) and manganese superoxide dismutase (MnSOD), both responsible for regulating ROS levels in cases of cellular oxidative stress. In the mitochondria, p66Shc, moves from the cytosol to the intermembrane space of the mitochondria, binding to cytochrome C and becoming an oxidoreductase that catalyzes the production of hydrogen peroxide (H_2O_2).

The ROS generated by these mechanisms will activate mitochondrial permeability transition pores, culminating in organelle dysfunction and in the release of mitochondrial apoptotic factors (caspases), in cell apoptosis and finally in the generation of glomerular endothelial dysfunction and sclerosis.[11, 12] Considering the information mentioned above, this study evaluated the potential use of *p66Shc* gene expression in liquid biopsy using urine sediment and peripheral blood, before changes in classic biomarkers, such as creatinine or microalbuminuria. For this, we used a biomarker of oxidative stress pathway that had already been studied by protein expression.

METHODS.

Design

The present study is a cross-sectional study. It was conducted in 2018 and early 2019, the patients were treated at the medical specialties' outpatient clinic of the *Centro Universitário FMABC/FMABC*. Patients who agreed to participate in the study were given a free and informed consent form (FICF). Blood samples were

not stored and were discarded after the measurements were made. We conducted an interview to collect the volunteers' personal data, as well as to measure height, body weight and verify any and all medications used to treat diabetes and its comorbidities.

Participants

The studied groups were as follows: Healthy individuals (CTL), healthy non-diabetic individuals without a family history of diabetes or kidney disease. The individuals who participated in this group were at least 21 years old, non-smokers or users of illicit drugs. Diabetic patients (T2DM), composed of patients diagnosed with type II diabetes Mellitus (fasting glucose ≥140mg/dL and glycated hemoglobin greater than 7%) for at least 5 years and preserved renal function (serum creatinine <1.3mg/dL and microalbuminuria <30mg/dL) with a minimum age of 21 years and undergoing treatment for T2DM. Exclusion criteria for T2DM group: Expressed will to participate by the patient; Diagnosed kidney disease (GFR <60mL/min/1.73m^2 or GFR> 60mL/min/1.73m^2) associated with at least one marker of parenchymal kidney damage (e.g. proteinuria> 15.0 mg/dL) present for at least 3 months. Exclusion criteria for T2DM group: Insulin dependent patient; Hospitalization for any reason in the last 30 days; Patient with a history of chronic liver disease.

This study was approved by the Ethics Committee of the Centro Universitário FMABC (no. 2.302.284). The informed consent forms were given to the volunteers for completion prior to their participation. The present study was carried out in accordance with the relevant guidelines and regulations/ethical principles of the Declaration of Helsinki.

Assessment of glycemic levels in patients with T2DM:

Determination of fasting plasma glucose was performed by assessing the concentration of glucose in the blood after a nocturnal fasting period. The automated enzymatic method was performed using fluoride serum. Evaluation of glycemic control was carried out with the values of fasting glucose, values above 140 mg/dL for glucose were considered altered.

Assessment of glycated hemoglobin (HbA1c) levels in patients with T2DM by LPLC:

Glycated hemoglobin (HbA1c) was determined using the low pressure liquid chromatography (LPLC) technique, using a DiaStat – Bio-Rad analyzer, which expresses the percentage of the total hemoglobin and evaluates the average blood glucose level, during a 3-month period.The collected material was 5 ml of whole blood with 1 ml of hemolyzed reagent. Values above 7% for HbA1c were considered altered.

Evaluation of kidney function in patients with DM2:

Serum creatinine was measured by the ELISA method to assess the kidney function of patients. The standard methodology of the Clinical Analysis Laboratory of the Faculdade de Medicina do ABC was followed. The estimated GFR was calculated by the Modification of Diet in Renal Disease (MDRD) formula.

Microalbuminuria determination

Determination of microalbuminuria in isolated urine samples was performed by the Biosystems® immunoturbidimetry method (BioSystems S.A. Costa Brava, Barcelona - Spain). Reference value was up to 15 mg/L for normoalbuminuric and between 30mg and 300 mg/24h for microalbuminuric.

Homocysteine quantification

Determination of total plasma homocysteine was performed by Abbott Diagnostics fluorescence polarization immunoassay. Plasma concentrations of total homocysteine were calculated by Abbott Axsym® and high values were considered to be those > 15 µmol/L, according to values proposed in a recent meta-analysis.

Cystatin C Quantification

Quantification of cystatin C was performed using the Enzyme Linked Immunosorbent Assay (ELISA) method, Cystatin C Kit (Human), catalog ALX-850-292, brand Enzo Life Sciences. This test is based on the identification of antigens by antibodies marked with an enzyme, which acts on its substrate and causes the color of the chromogen (colorless substance that when oxidized by the enzyme causes a change in its color) to change.

p66Shc gene expression in peripheral blood cells and urinary sediment cells

Extraction of total RNA in peripheral blood cells: Total RNA was isolated from leukocytes contained in peripheral blood obtained through hemolysis by centrifugation at 2500 RPM for 15 minutes, using the TRIzol method (TRIzol LS Reagent, Thermo Fisher cat. no. 10296-010), according to the manufacturer's protocol. To extract total RNA from the urine sediment: Samples (15 mL) were initially centrifuged at 2500 revolutions per minute (RPM) for 10 minutes at 4ºC to obtain the urine sediment. The supernatant was discarded and 1 ml of TRIzol was added to the cell pellet. The extraction process followed the standard protocol instructions for TRIzol. Total RNA concentration was estimated by spectrophotometric reading using a NanoDrop equipment (ThermoFisher Scientific - Waltham, Massachusetts, USA). cDNA synthesis, samples of total RNA (starting amount 1µg) obtained from peripheral blood and urine sediment were converted into cDNA using *SSIII First Strand qPCR Supermix* (Invitrogen, cat. no. 11752050), according to the manufacturer's protocol. RT-qPCR, p66Shc gene expression was evaluated by real-time PCR (RT-qPCR). The specific primers for each selected gene were designed with the aid of the Primer3 Input 0.4.0 software program, available at http://frodo.wi.mit.edu/primer3/. The designed primer sequences were then checked for specificity by the *Primer-BLAST* program, available at http://www.ncbi.nlm.nih.gov/tools/primer-blast. To normalize the relative expression of the target genes, expression values of the reference gene *RPL13A* were used.

Sequence of specific primers and their amplicons.

	Forward	Reverse	pb
p66Shc	GCTGCATCCCAACGACAAAG	GAGTCCGGGTGTTGAAGTCC	113
RPL13A	TTGAGGACCTCTGTGTATTTGTCAA	CCTGGAGGAGAAGAGGAAAGAGA	126

Statistical analysis

The results were expressed as mean±standard deviation (SD). These were compared using unpaired Student's *t*-test, and Mann-Whitney for nonparametric data. These analyses were performed with the aid of the computer program GraphPad Prism (GraphPad, version 7.0, USA). The significance level was set at 5% (descriptive $p < 0.05$). The sample size was determined by calculations performed in the computer program GPower 3.1.

RESULTS.

A total of 66 volunteers were evaluated, of which 37 healthy participants - CTL and 29 patients with T2DM. Within the CTL group, there were 55% female participants and 45% male participants. The mean age was 45±14 years with a predominance of Caucasian ethnicity (89%), and 5% of the total were hypertensive (Table 1).

(Table 1)

In the T2DM group, we obtained 60% of female participants against 40% of male sex. The mean age was 63±8 years and the predominant ethnicity was Caucasian (75%). The majority of this population reported having arterial hypertension (60%), 20% were not hypertensive and another 20% reported not knowing this information. When we evaluated the time since diagnosis of T2DM, we found that 80% of participants had been diabetic for at least 5 years, 5% between 5 and 10 years and 15% for more than 10 years (Table 2).

(Table 2)

To characterize the studied population, we performed blood glucose measurements of the CTL and T2DM groups (87±11 vs. 152±71 mg/dL, *p<0.05) as well as Hb1Ac (5.5±0.4 vs. 7.5±1.9%, *p<0.05) respectively. We were able to observe a statistical difference between the groups due to high values of these biochemical markers, as we expected. BMI values were compared between groups (CTL: 27±5 vs. T2DM: 28±5 Kg/m^2) (Figure 1).

(Figure 1)

Figure 2 shows the evaluations of classic biochemical markers of renal function. We did not observe alterations in the values of plasma creatinine (A) (CTL 0.80±0.20 vs. T2DM 0.87±0.29 mg/dL), urinary creatinine (B) (CTL 134±85 vs. 130±69 mg/dL), Urea (C) (CTL 32±12 vs. T2DM 53±83 mg/dL), proteinuria (D) (CTL 12.2±10.2 vs. 23.1±48.1 mg/dL) and GFR (F) data. We only verified alterations in the values of microalbuminuria (D) (CTL 20.3±37.0 vs. T2DM 23.4±24.7 mg/L, *p<0.05).

(Figure 2)

Figure 3 illustrates *p66Shc* gene expression in blood (A) and urine (B) samples. We observed that there was no statistical difference in the expression of this gene between the CTL group and T2DM group. However, *p66Shc* expression in blood was 3.6 times higher in diabetics (T2DM 0.024±0.079) than in healthy participants (CTL 0.0069±0.0176 $^{2-\Delta CT}$). In urine, *p66Shc* expression was 1.48 times higher in diabetics (0.0276±0.0541) than in CTL (0.0186±0.0219 $^{2-\Delta CT}$).

(Figure 3)

When measuring Hcy and cystatin C concentrations, we did not identify significant alterations between groups: Hcy T2DM (22.20±4.15 µmol/L) and CTL (19.90±5.61 µmol/L), cystatin C (T2DM 1.05±0.15 vs. CTL 1.02±0.14 mg/dL) (Figure 4).

(Figure 4)

DISCUSSION.

The concept of liquid biopsy is based on the use of liquid/fluid samples (especially from peripheral blood or urine) to detect early changes in the expression of a gene of choice, through the evaluation of cell-free nucleic acids. This is a new approach that has already been studied in cancer and is able to indirectly reflect the future expression of proteins involved in the installation of tumors or changes in tissue function. Furthermore, the fundamental idea of this method is to be less invasive than traditional disease probing methods. This method still needs to be standardized for the consolidation of its diagnostic and/or prognostic use, and therefore the efforts for this elucidation are valid. This study evaluated a biomarker that actively participate in the oxidative stress pathway, to proposing an early marker of renal changes in diabetics patients.[13-15]

Oxidative stress in diabetic patients is higher when compared to healthy patients, since the formation of intracellular ROS - considered a common route in renal injury induced by hyperglycemia - is greater in these patients and increases proportionally with the development of the disease.[16] As previously described, p66Shc is a protein responsible for modulating the production of mitochondrial ROS, causing the stressed cell to produce more ROS, providing positive feedback that will result in cell apoptosis. Despite the studies identifying this activation of p66Shc associated with the pathophysiology of DN in endothelial cells, this study evaluated whether this alteration also occurred in peripheral blood leukocytes and urinary sediment cells, in view of the ease and importance of using liquid biopsy for monitoring of patients. Liquid biopsy, originally studied in oncology, consists of isolating circulating cells as a source of genomic and proteomic information.[17]

The increase in *p66Shc* gene expression was not significant. However, looking at our results from a different analysis, we found that the *p66Shc* gene expression in the blood was 3.6 times higher in diabetics compared to healthy individuals, in urine, this difference was 1.48 times greater under the same comparison. This could suggest that in a condition with a higher number of patients, we would see a significant increase in this gene expression. The study of *p66Shc* gene expression in diabetic patients without diagnosed kidney injury had not been explored, at least to our knowledge.

Our data showed that all diabetic patients had high levels of glycemia and glycated hemoglobin, adequately characterizing the state we wanted to investigate. In parallel to this, we carried out an evaluation of classic markers of kidney disease onset, creatinine, proteinuria, microalbuminuria and the calculation of GFR, in all these measurements we observed that our patients did not have kidney disease prior to the evaluations.

As is known, cystatin C is a current and accurate marker for the assessment of initial renal function loss, as it is freely filtered by the glomerulus and subsequently reabsorbed in the proximal tubule, so its determination in serum reflects glomerular filtration, and its increase in serum means a reduction in GFR.[18] Our patients did not show an increase in the measurement of this marker, confirming that our diabetic group did not have nephropathy.

The participants with T2DM did not show an increase in Hcy measurements, which evidenced the absence of endothelial injury. In our study there was a subtle alteration in *p66Shc* gene expression, without significant changes in Hcy levels. The relationship between *p66Shc* overexpression and increased Hcy levels has been previously described in patients with confirmed endothelial dysfunction. The main cause of this relationship is closely linked to DNA methylation, promoted by *p66Shc.*[19, 20] Our data suggest the increase in

p66Shc expression is the mechanism responsible for the initiation of deregulation of Hcy synthesis. We believe that the consolidation of the overexpression of this gene increases DNA methylation and causes, in later stages of T2DM, irreversible endothelial changes, followed by kidney damage. On the other hand, when there are high levels of the Hcy precursor, S-Adenosilhomocysteine (SAH), there is an increase in the production of ROS and in the expression of p66Shc in endothelial cells, that is, there is self-regulation between the expulsion of this gene and the increase in Hcy.[21]

The relationship between *p66Shc* expression and diabetic nephropathy is evident, studies in mice have shown that deletion of this gene prevents endothelial dysfunction induced by hyperglycemia, in addition to reducing the oxidative stress of cells, which prevented the alteration of renal structure and function in these animals.[22-24] The endothelial and myoblastic cells of *p66Shc* knockout mice demonstrated a lower rate of apoptosis in ischemic conditions, thus proving the role of p66Shc in cell survival in response to hypoxia.[25] Another protein involved in renal damage to DN is the hypoxia-inducible factor (HIF-1α). The relationship between p66Shc and HIF-1α has been described by proposing a pathway in which HIF-1α, stimulated by T-cell hypoxia, activates p66Shc which contributes to the release of extracellular vascular endothelial growth factor (VEGF), being one response to low oxygenation mediated by HIF-1α. In addition, p66Shc itself is stimulated by oxidative stress induced by hypoxia, and triggers cell apoptosis.[26] Our study identified the subtle elevation of *p66Shc* gene expression in diabetic patients who, as previously described, are in oxidative imbalance due to hyperglycemia, hyperinsulinemia and insulin resistance and thus, we can suggest that the pathway explained above is starting to be activated.[9]

Expression of the *p66Shc* protein in peripheral blood and in renal tissue of diabetic patients had already been studied, however, under conditions of already established kidney injury. The authors found an increase in the expression of the p66Shc protein and suggested that the evaluation of its expression in peripheral blood could be used as a potential biomarker of the progression of kidney injury mediated by increased oxidative stress.[27]

Considering that the oxidative stress pathway is activated by hyperglycemia, we suggest that in our patients there is a synergism between the hyperexpression of *mothers against decapentaplegic homolog 1* (*SMAD1*), which are intracellular proteins capable of regulating transcription factors and expression of target genes, associated with that of *p66Shc*, with the first promoting mesangial expansion and the second mediating oxidative imbalance. Positive feedback between these two pathways may be responsible for the gradual and silent loss of kidney function. Therefore, we suggest that minor changes in *p66Shc* gene expression may signal the dysregulation of the oxidative system and, thus, lead to late kidney damage.[21, 23, 28, 29]

This study showed that there was no significant *p66Shc* gene expression in peripheral blood and urine samples of diabetic patients without kidney injury in comparison with health donors, however, in our experimental conditions *p66Shc* gene expression is slightly increased in T2DM group. There was no association between increased gene expression and the other laboratory variables that were studied. We believe that increasing the number of patients may elucidate the viability of the data.

The limitation of this study was the low sample size and the difficulty in assessing gene expression in urine samples. We are certain that the use of specific extraction kits for samples of low cellularity, such as urine samples, will facilitate this type of study in this biological matrix.

Summary - Accelerating Translation (Portuguese)

Título: Estudo transversal da expressão do gene p66Shc em biópsia líquida de pacientes diabéticos. É possível prever o início da doença renal?

O objetivo deste estudo foi avaliar a expressão do gene *p66Shc* como um possível biomarcador precoce de disfunção renal em pacientes com diabetes, utilizando amostras líquidas de sedimento urinário e sangue periférico. Foram avaliados 29 pacientes diabéticos e 37 doadores saudáveis, estes foram recrutados no ambulatório do Centro Universitário FMABC. Foi avaliada a expressão do gene p66Shc pela técnica de RT-qPCR em amostras de urina e sangue periférico de pacientes diabéticos e foram comparadas com doadores saudáveis. Não foi observado alteração da expressão do gene p66Shc em amostras de pacientes diabéticos em comparação com doadores saudáveis. No entanto, a expressão de p66Shc no sangue de diabéticos foi 3,6 vezes maior em diabéticos (0,02417±0,078652-ΔCT, n=29) do que em participantes saudáveis (0,00689±0,01758, n=37) e na urina foi 1,48 vezes maior no grupo de diabéticos (0,02761±0,05412-ΔCT) do que no grupo CTL (0,0186±0,02199). Portanto, não houve expressão significativa do gene *p66Shc* em amostras de sangue periférico e urina de pacientes diabéticos sem lesão renal em comparação com doadores saudáveis, embora haja uma tendência desse gene participar do desequilíbrio oxidativo presente no diabetes.

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FIGURES AND TABLES.

Figure 1. Values of blood glucose (A), glycated hemoglobin (Hb1Ac) (B) and body mass index (BMI) (C) of healthy participants (CTL) versus diabetic participants (T2DM). Values expressed as mean±SD. *p<0.05 vs. CTL. Unpaired Student's *t*-test.

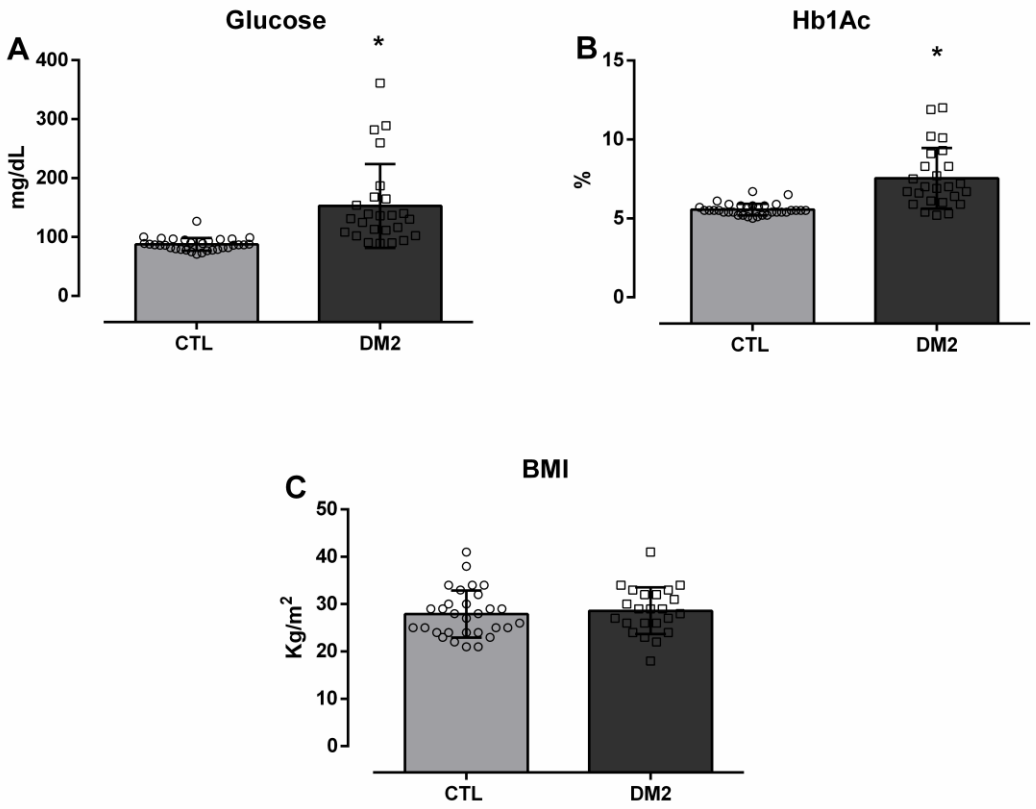

Figure 2. Values for plasma creatinine (A), urinary creatinine (B), urea (C), microalbuminuria (D), proteinuria (E) and glomerular filtration rate (GFR) (F) of healthy participants (CTL) versus diabetic participants (T2DM). Values expressed as mean±SD. *p<0.05 vs. CTL. Unpaired Student's *t*-test.

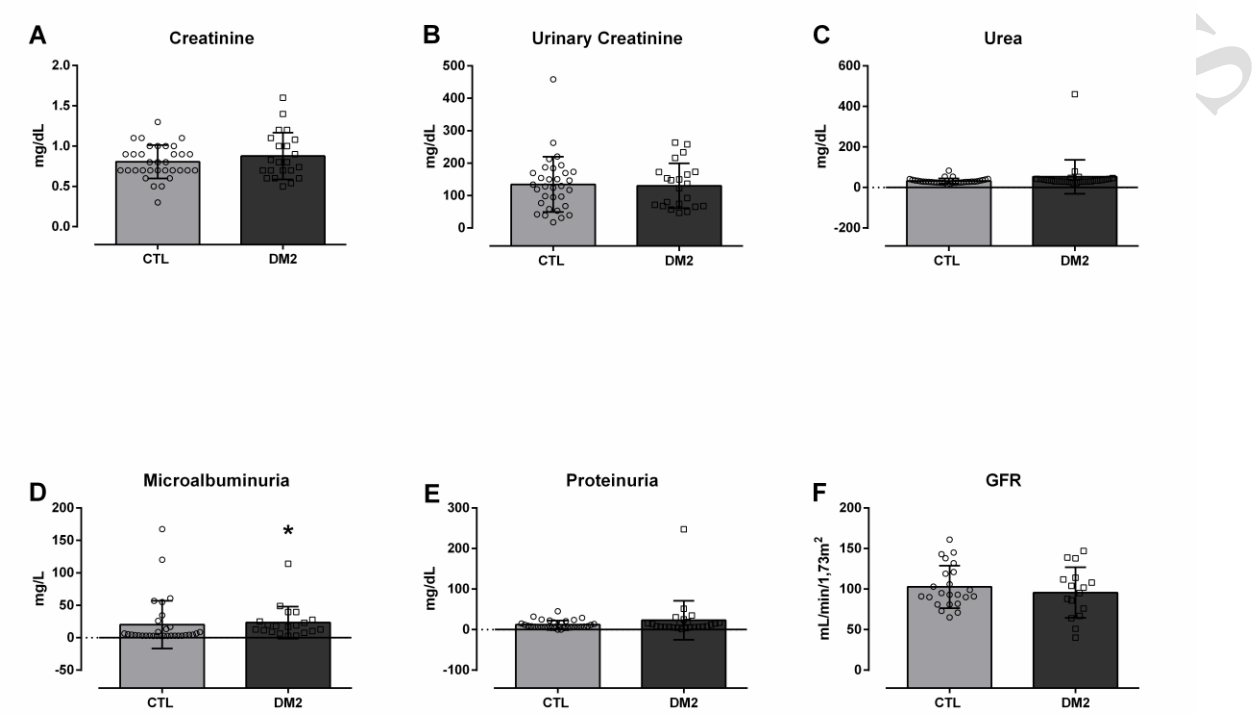

Figure 3. p66Shc gene expression values ($^{2-\Delta CT}$) in healthy participants (CTL) compared to diabetic participants (T2DM) in blood (A) and urine (B) samples. Mann-Whitney test.

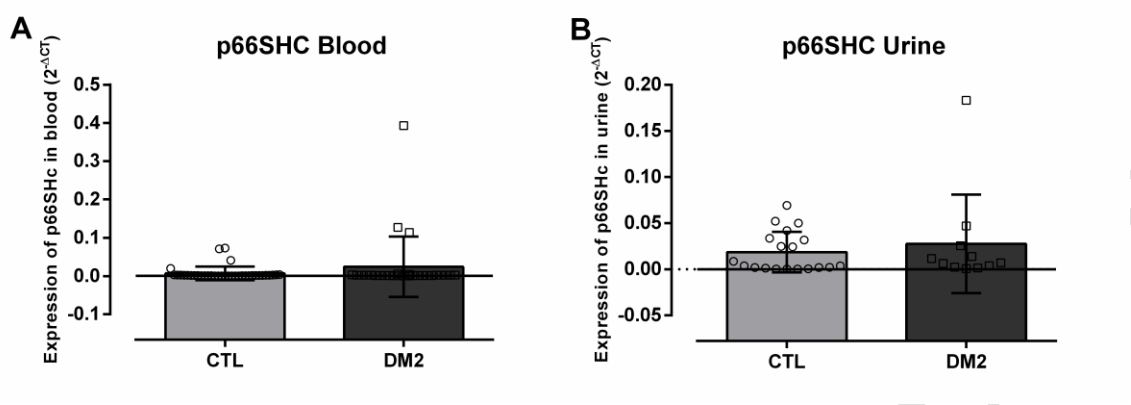

Figure 4. Representative graphs referring to the measurement of homocysteine (A) and Cystatin C (B) of healthy participants (CTL) versus diabetic participants (T2DM). Values expressed as mean±SD. *p<0.05 vs. CTL. Mann-Whitney test.

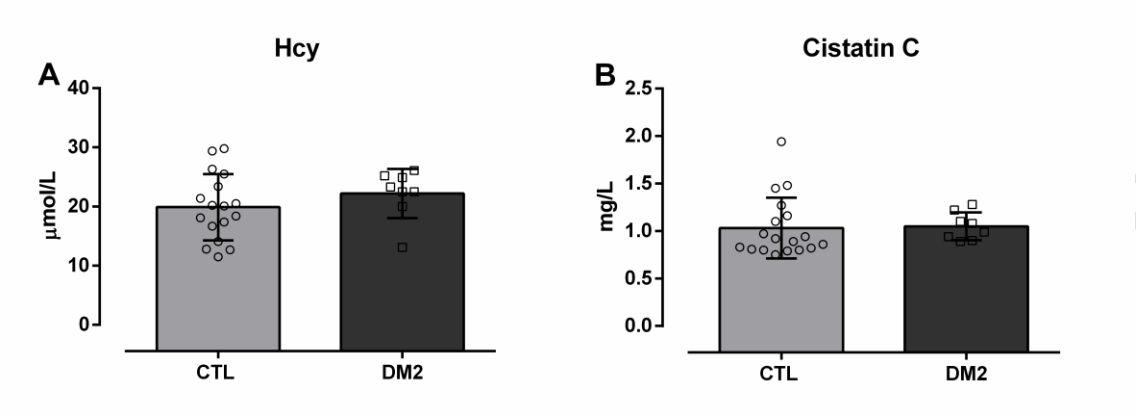

Table 1. Anthropometric data of participants in the healthy group (CTL)

Parameters	
Gender (%)	
Female	55
Male	45
Age (mean±SD) years	45±14
Ethnicity (%)	
Black/Brown	11
Caucasian	89
Arterial Hypertension (%)	
Yes	5
No	95
Don't know	0

Note: Standard Deviation (SD).

Table 2. Anthropometric data of participants in the diabetic group (T2DM)

Parameters	
Gender (%)	
Female	60
Male	40
Age (mean±SD) years	63±8
Ethnicity (%)	
Black/Brown	25
Caucasian	75
Arterial Hypertension(%)	
Yes	60
No	20
Don't know	20
Time of disease (%)	
0-5 years	80
5-10 years	5
>10 years	15

Note: Standard Deviation (SD).