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Poor glycaemic control in type 2 diabetes compromises leukocyte oxygen consumption rate, OXPHOS complex content and neutrophil-endothelial interactions

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ABSTRACT

The mitochondrial electron transport chain becomes overloaded in type 2 diabetes (T2D), which increases ROS (Reactive Oxygen Species) production and impairs mitochondrial function. Peripheral blood mononuclear cells (PBMCs) are critical players in the inflammatory process that underlies T2D. Poor glycaemic control in T2D is closely linked to the development of comorbidities.

Our aim was to evaluate if glycaemic control in T2D has an impact on the oxygen consumption rates (OCR) of PBMC, OXPHOS complexes and inflammation.

We recruited 181 subjects, consisting of 79 healthy controls, 64 patients with T2D and good glycaemic control (HbA1c<7 %), and 38 T2D patients with poor glycaemic control (HbA1c>7 %).

We found a decrease in the basal OCR of PBMCs from patients with HbA1c>7 % with respect to controls ($p < 0.05$). Maximal OCR and spare respiratory capacity were lower in patients with HbA1c>7 % than in controls and patients with HbA1c<7 % ($p < 0.05$ for all). Mitochondrial ROS levels were higher in T2D patients, and particularly in the HbA1c > 7 group ($p < 0.05$ HbA1c<7 % vs control, $p < 0.001$ HbA1c>7 % vs control; $p < 0.001$ HbA1c > 7 vs HbA1c < 7). With respect to controls, poor glycaemic control in T2D patients was associated with a decrease in mitochondrial complex III and V ($p < 0.05$ and $p < 0.01$, respectively) and enhanced neutrophil-endothelial interactions ($p < 0.001$ vs controls). MPO levels were enhanced in T2D patients in general ($p < 0.05$ vs controls), and ICAM-1 and VCAM-1 were specifically increased in HbA1c > 7 patients vs controls ($p < 0.01$ and $p < 0.001$, respectively). Negative low-to-moderate correlations were found between HbA1c and basal respiration ($r = -0.319$, $p < 0.05$), maximal respiration ($r = -0.350$, $p < 0.01$) and spare respiratory capacity ($r = -0.295$, $p < 0.05$).

Our findings suggest that poor glycaemic control during the progression of T2D compromises mitochondrial respiration and OXPHOS complex content in PBMCs. These alterations occur in parallel to enhanced neutrophil-endothelial interactions and adhesion molecule levels, leaving T2D patients with poor glycaemic control at a higher risk of developing vascular diseases.

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1. Introduction

Non-communicable diseases, such as obesity, type 2 diabetes (T2D) and cardiovascular diseases, which are often preventable disorders, are the leading causes of death and disability worldwide [1]. Their prevalence has been increasing steadily over the past few decades; in the case of diabetes, 1.5 million deaths are directly attributed to it each year [2]. T2D is a chronic metabolic disorder characterized by hyperglycaemia due to defective insulin action. Excessive reactive oxygen species (ROS) are a direct consequence of hyperglycaemia and result in damage to various tissues, including blood vessels and nerves [3]. These alterations result in the development of diabetic complications such as cardiomyopathy, peripheral vascular disease and atherosclerosis, among others. Furthermore, poor glycaemic control - i.e., the inability to preserve adequate blood glucose levels, despite treatment - is strongly associated with the development of these comorbidities [4]. The gold standard biomarker for diabetes diagnosis is haemoglobin A1c (HbA1c), an indicator of glycaemic control on which clinicians rely for monitoring a patient's response to medical treatments [5].

Leukocytes are critical players in the inflammatory process and in the development of atherosclerosis. Interestingly, leukocyte bioenergetics profile determination provides a translational tool to understand the mitochondrial mechanisms of human systemic pathogenesis [6]. Basal release of ROS is essential for physiological functions in the immune system. For example, mitochondrial membrane hyperpolarization and ROS production play a key function during immune activation of monocytes and lymphocytes [7]. However, chronic ROS hyper-production is a hallmark of pathological processes that underlie metabolic diseases, such as diabetes [8,9]. Mitochondrial OXPHOS complex I and III are responsible for ROS production under physiological and pathological conditions [10]. In this sense, it has been previously been shown that complex I function is impaired in the leukocytes of T2D patients, which display reduced mitochondrial O_2 consumption and increased ROS production [11].

Alterations in leukocyte oxygen consumption rates (OCR), especially in the peripheral blood mononuclear cells (PBMCs) of individuals with T2D, have previously been reported in several studies [12–14]. However, results are contradictory, with some studies reporting decreased mitochondrial respiration in subjects with T2D [12,14] and others observing increased OCR [13].

Prior to beginning the present study, we hypothesized that the condition of T2D can negatively affect PBMC OCR, and that glycaemic control of the disease has an impact on PBMC bioenergetics. In addition, we suspected that these disturbances may be associated with the classic inflammatory phenotype of T2D. Based on these assumptions, we evaluated OCR in subjects with T2D, including those with good and poor glycaemic control, and compared it to values in a healthy population. In addition, we evaluated the levels of mitochondrial complexes, mitochondrial ROS production, and the inflammatory process by assessing levels of pro-inflammatory cytokines, adhesion molecules and neutrophil-endothelial interactions in T2D patients with good glycaemic control (HbA1c<7 %), poor glycaemic control (HbA1c>7 %), and their respective controls.

2. Material and methods

2.1. Subjects and samples collection

79 healthy control subjects and 102 patients with T2D - diagnosed following the guidelines of the American Diabetes Association (ADA) - were recruited at the Endocrinology Service of University Hospital Dr. Peset (Valencia, Spain). The study was approved by the Ethics Committee for Clinical Investigation of University Hospital Dr. Peset (ID: 100.22) and complied with the principles of the Helsinki declaration. Participants were informed of the aims of the study and gave their written informed consent. T2D patients were separated into poor glycaemic control (HbA1c>7 %, n = 38) and good glycaemic control (HbA1c<7 %, n = 64) groups. The threshold value of HbA1c was based on studies showing that HbA1c < 7 prevents the incidence of macro- and microvascular comorbidities [15–17]. After 12 h of fasting, anthropometric parameters were obtained and peripheral blood was drawn.

2.2. Biochemical parameters

Venous blood was collected into Vacutainer® tubes in a fasting state between 8 a.m. and 9 a.m., during which time patients also underwent a physical examination to determine weight and height. Biochemical and molecular parameters were measured through blood sampling at our hospital's Clinical Analysis Service. Glucose, triglycerides and total cholesterol levels in serum were measured by means of an enzymatic method. Insulin levels were calculated by immunochemiluminescence and insulin resistance was measured by homeostasis model assessment (HOMA-IR = [fasting insulin (μU/mL) × fasting glucose (mg/dL)]/405). HbA1c percentage was determined with a glycohaemoglobin analyzer (Arkray Inc., Kyoto, Japan) and high-sensitive C-reactive protein (hs-CRP) levels were evaluated by an immunonephelometric assay. High-density lipoprotein cholesterol (HDL-c) was determined with a Beckman LX-20 autoanalyser (Beckman Coulter, La Brea, CA, USA) and low-density lipoprotein cholesterol (LDL-c) was quantified using Friedewald's formula.

The total antioxidant capacity of the subjects' serum was assessed using a commercial kit (OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit, STA-360, Cell Biolabs, Inc.). To measure 4-HNE adduct in serum, we used the OxiSelect™ HNE adduct competitive ELISA kit (Cell Biolabs, Inc.), and the amounts of carbonyl groups in serum proteins were determined using the OxiSelect™ Protein Carbonyl ELISA kit (Cell Biolabs, Inc.). All kits were performed according to the manufacturers' protocols.

2.3. Isolation of PBMCs and neutrophils

PBMCs and neutrophils were isolated from whole blood of all participants, collected in EDTA-coated tubes. PBMCs were isolated using the MACSprep™ kit (130-115-169, Miltenyi Biotec) following the manufacturer's guidelines. In the case of neutrophils, negative immunomagnetic selection was performed with the MACSxpress Whole Blood Neutrophil Isolation kit (130-104-434, Miltenyi Biotec), following the manufacturer's protocol. Cell density was measured with the cell counter LUNA-FL™ (Logos Biosystems).

2.4. Measurement of oxygen consumption rate

Cellular oxygen consumption rate (OCR) was estimated using the Agilent Seahorse XFp Analyzer. Extracellular Flux Cartridges (Agilent Technologies) were hydrated and Cell Culture Miniplates (Agilent Technologies) were treated with poly-D-lysine one day prior to seeding with PBMCs (300,000 cells/well). Absolute OCR, which is an indicator of the functional state of cellular mitochondria, was measured using the Agilent Seahorse XFp Analyzer and the Seahorse XFp Cell Mito Stress Test Kit (103,010–100, Agilent Technologies), following the manufacturer's instructions.

2.5. Flow cytometry assay

After erythrocyte lysis with RBC Lysis Solution (MACS, Cat. number 130-094-183, Milteny Biotech, Germany), 200 μL of human PBMCs were labeled with 5 μL of APC-CD45 antibody (BD pharmingen APC-mouse anti human CD45. CAT number 555485 BD biosciences, NJ, USA) and 1 μM MitoSox (Thermo-Fisher Scientific, MA, USA). Fluorescence was measured in a C6 Accuri cytometer (BD Biosciences, NJ, USA) with a blue laser (488 nm) and FL3 filter (585/40 nm) (mitoSOX Ex/Em = 510/580). 10,000 cells were analyzed in each experiment, and the

fluorescence registered was relativized to that of an internal control consisting of U937 cells that had undergone the same protocol [8,18].

2.6. Western blot

Protein extraction from PBMCs was performed with Pierce RIPA buffer (89800/89901, Thermo Scientific). Protein quantification was carried out with the Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific). 25 μg of proteins were separated onto Novex™ 4–20 %, Tris-Glycine Plus Wedge Well™ gel (XP04205BOX, Thermo Fisher Scientific) at 150V for 1 h. The proteins were transferred to a nitrocellulose membrane (0.45 μm, 1620167, BioRad) at 400 mA for 1 h. Subsequently, membranes were incubated with a primary antibody against OXPHOS (1:1000, ab110411, Abcam) and VDAC (1:1000, ab14734, Abcam), and with anti-mouse (1:2000, 31430, Invitrogen). The chemiluminescent signal was produced with Super Signal West Pico (34580, Thermo Fisher Scientific) to obtain images through Fusion FX and was processed using the Bio1D software. All results were normalized with respect to VDAC protein expression.

2.7. Neutrophil-endothelial cell interaction

Human umbilical vein endothelial cells (HUVEC-TERT2 CRL-4053, ATTC) were cultured on a monolayer until confluence in 35 mm × 10 mm culture dishes (430,165, Corning Incorporated) with RMPI media supplemented with 10 % v/v FBS (Biowest). HUVECs were treated with 1.25 ng/mL TNF-α for 4 h before starting the experiment. Neutrophils were resuspended (1.2×10^6 cell/mL) in RPMI media supplemented with 10 % v/v FBS (Biowest) and perfused over the HUVEC monolayer for 5 min at a speed of 3.3 mL/min using a dynamic adhesion system with a parallel flux chamber. They were recorded with a Nikon Eclipse Ts2R microscope. Rolling velocity, rolling flux and adhesion parameters were calculated with Tracker software.

2.8. Evaluation of adhesion and inflammatory molecules

Blood in serum separator tubes was centrifuged (1500g for 10 min at 4 °C) in a Luminex® 200 (Luminex Corporation) to analyze serum levels of the adhesion molecules ICAM-1, P-selectins, VCAM-1, myeloperoxidase (MPO) and cytokines IL6, TNF-α, IL12 and IL10, following the MILLIPLEX® Human High Sensitivity T Cell Magnetic Bead Panel (HSTCNAG-28SK) and MILLIPLEX Human Cardiovascular Disease (CVD) Magnetic Bead Panel 2 (HCVD2MAG-67K) manufacturer's procedures (Merck KGaA Corporation).

2.9. Statistical analysis

Normal distribution of data samples was confirmed with the Shapiro-Wilk test. Normally distributed data were compared with a *t*-test and non-normally distributed data were compared with a Mann Whitney *U* test. One-way ANOVA and a Tukey post-hoc test were employed to compare the 3 groups. The Chi-square test was used to compare proportions, and the influence of sex, age and BMI was tested by a general linear model. Spearman's correlation coefficient was used for correlation studies to compare clinical parameters and protein expression levels.

These analyses were performed using SPSS (SPSS Statistics Inc.) and Prism 9.0.2 (www.graphpad.com).

3. Results

3.1. Metabolic profile

Table 1 summarizes the metabolic and anthropometric parameters of the different study groups. There was a higher proportion of women in the control group (63.4 %) than among the T2D patients (HbA1c < 7 = 36.1 %, HbA1c > 7 = 39.4 %; $p < 0.05$). T2D patients were older (HbA1c < 7 = 57.2 ± 8.8; HbA1c > 7 = 60.4 ± 10.1 years old) and had a higher BMI (HbA1c < 7 = 28.6 ± 2.96; HbA1c > 7 = 27.8 ± 3.59 kg/m^2) than the control group (age = 48.2 ± 10 years old; $p < 0.001$, BMI = 22 ± 1.17 kg/m^2; $p < 0.05$). In addition, typical metabolic alterations were observed in the T2D population, including elevated glucose ($p < 0.001$) and HOMA-IR ($p < 0.05$), and dyslipidaemia, reflected by increased triglycerides ($p < 0.01$) and decreased HDL-c levels ($p < 0.001$). Total cholesterol and LDL-c values were reduced in subjects with T2D ($p < 0.001$), probably because most of them (82 %) were under statin treatment (Supplementary Table 1). Interestingly, chronic low-grade inflammation was enhanced in T2D subjects, estimated as hs-CRP ($p < 0.05$) and leukocyte counts ($p < 0.001$) (in particular, neutrophils ($p < 0.001$), lymphocytes ($p < 0.01$) and monocytes ($p < 0.01$)). Significant differences remained for glucose ($p < 0.05$), HbA1c ($p < 0.001$) and monocyte count ($p < 0.05$) after adjusting for age, sex and BMI. The monocyte-to-lymphocyte ratio was similar among the different groups.

Table 1
Summary of anthropometric and biochemical parameters.

Characteristics	Control	HbA1c < 7	HbA1c > 7	p-value	Sex, age and BMI- adjusted p-value
N	79	64	38		
Sex (% women)	63.4	36.1*	39.4	p < 0.05	
Age (years)	48.2 ± 10.0	57.2 ± 8.8***	60.4 ± 10.1***	p < 0.001	
BMI (kg/m^2)	22.0 ± 1.17	28.6 ± 2.96*	27.8 ± 3.59	p < 0.05	
Glucose (mg/dl)	89.2 ± 10.5	112 ± 23.7**	155 ± 57.2***$^{\#\#\#}$	p < 0.001	p < 0.05
HOMA-IR	1.79 ± 1.31	2.9 ± 1.7**	2.74 ± 2.34	p < 0.05	ns
Insulin (μUl/mL)	8.05 ± 5.23	10.5 ± 6.9	7.41 ± 5.0	ns	ns
HbA1c (%)	5.17 ± 0.32	6.18 ± 0.50***	7.91 ± 1.07***$^{\#\#\#}$	p < 0.001	p < 0.001
HbA1c (mmol/mol)	32.7 ± 4.94	44 ± 5.4***	63.1 ± 11.7***$^{\#\#\#}$	p < 0.001	p < 0.001
hsCRP (mg/L)	0.78 (0.39–2.48)	1.21 (0.63–3.60)	2.06 (0.91–5.6)	p < 0.05	ns
Total cholesterol (mg/dL)	200 ± 40.4	155 ± 44.4***	148 ± 36.4***	p < 0.001	ns
HDL-c (mg/dL)	58.1 ± 16.7	48.3 ± 12.4***	45.2 ± 10.4***	p < 0.001	ns
LDL-c (mg/dL)	123 ± 37.2	75.2 ± 36.4***	73.2 ± 33.9***	p < 0.001	ns
Triglycerides (mg/dL)	82 (56–116)	111 (77–163)*	121 (83–202)*	p < 0.01	ns
Leukocyte count (x10^9/l)	5.92 ± 1.35	7.00 ± 2.12*	8.32 ± 2.76***$^{\#}$	p < 0.001	ns
Neutrophil count (x10^9/l)	3.43 ± 1.02	4.18 ± 1.65	5.14 ± 2.23***$^{\#}$	p < 0.001	ns
Lymphocyte count (x10^9/l)	1.79 ± 0.52	2.06 ± 0.63	2.29 ± 0.76**	p < 0.01	ns
Monocyte count (x10^9/l)	0.479 ± 0.162	0.548 ± 0.171	0.636 ± 0.212***	p < 0.01	p < 0.05
Eosinophil count (x10^9/l)	0.187 ± 0.145	0.212 ± 0.18	0.194 ± 0.143	ns	ns
Monocyte-to-lymphocyte ratio	0.248 ± 0.113	0.287 ± 0.129	0.293 ± 0.109	ns	ns

Data expressed as means ± standard deviation or median and interquartile range. *p < 0.05; **p < 0.01; ***p < 0.001 vs Control. $^{\#}$p < 0.05; $^{\#\#}$p < 0.01; $^{\#\#\#}$p < 0.001 vs HbAc1<7.

A summary of the treatments received by T2D patients is provided in Supplementary Table 1.

3.2. Evaluation of OCR and mitochondrial ROS production of PBMCs

To estimate the OCR of the cells, we performed Seahorse extracellular flux analysis of the different groups. Our results showed a significant decrease in basal OCR in T2D HbA1c > 7 patients compared to the control and T2D HbA1c < 7 groups (Fig. 1A–B, $p<0.05$). Maximal respiration and spare respiratory capacity were significantly reduced in patients with poor glycaemic control (Fig. 1C and E, $p < 0.05$). A significant decrease in maximal respiration and spare respiratory capacity was observed in the T2D HbA1c > 7 versus T2D HbA1c < 7 group (Fig. 1C, $p < 0.05$). Another notable finding was a significant increase in non-mitochondrial respiration in T2D HbA1c < 7 patients compared to controls (Fig. 1F, $p < 0.05$). These findings indicate that patients with poor metabolic control exhibit reduced OCR, suggesting a decrease in the activity of OXPHOS complexes, which would lead to mitochondrial dysfunction. Despite these observations, no significant differences were detected in ATP production among the different study groups.

In addition, we estimated mitochondrial ROS by Mitosox Red staining, detecting higher mitochondrial ROS production in PBMCs of T2D patients with good metabolic control (Fig. 1G, $p < 0.05$ in T2D HbA1c < 7) and poor metabolic control ($p < 0.001$ in T2D HbA1c > 7) than in those of control subjects, and more so in the latter group (HbA1c > 7) ($p < 0.001$) (Fig. 1G). When we employed TMRM fluorescence to explore mitochondrial membrane potential in a subset of individuals from the different study groups, we found no difference among the 3 groups, ruling out the possibility that the Mitosox Red signal was caused by mitochondrial hyperpolarization (Supplementary Fig. 1).

To better evaluate whether increased mitochondrial ROS in PBMCs is manifested as systemic oxidative damage we assessed total antioxidant capacity, 4-HNE and protein carbonyl groups in serum. No differences in these parameters were found among the groups (Supplementary Fig. 2).

3.3. OXPHOS complexes content

Next, we performed Western Blot analysis to determine the protein levels of the different OXPHOS complexes. Our results revealed no significant differences in the protein levels of complexes I, II, and IV among the groups (Fig. 2A, B and 2D). However, a significant decrease in complex III (Ubiquinol-cytochrome c reductase, Fig. 2C, $p < 0.05$) and complex V (ATP synthase, Fig. 2E, $p < 0.01$) was observed in the T2D HbA1c > 7 group. These findings confirm an unbalanced mitochondrial bioenergetics components in patients with poor glycaemic control of the disease.

3.4. Neutrophil-endothelial interactions

The development of the inflammatory process associated with T2D

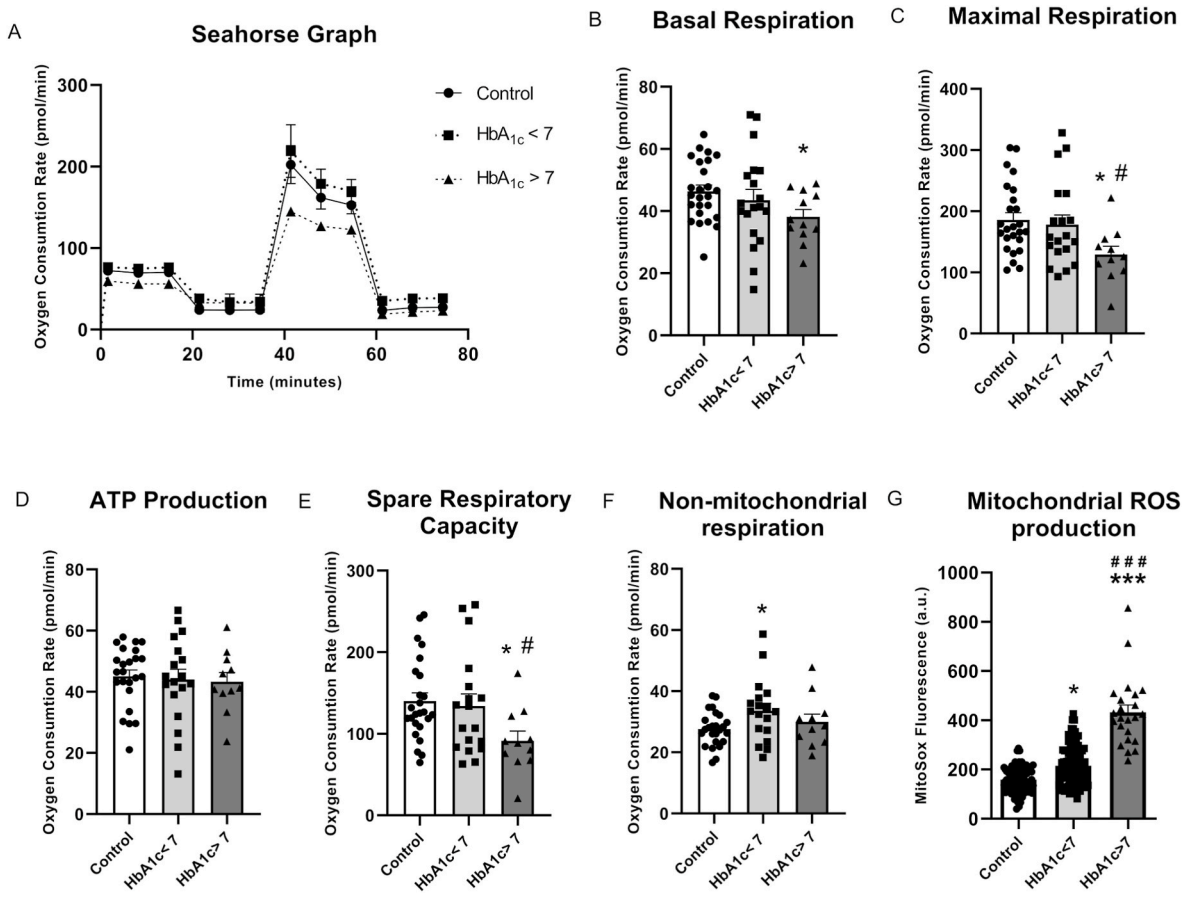

Fig. 1. Mitochondrial function in PBMCs from control individuals and subjects with T2D and poor or good glycaemic control. A) Representative OCR during Mito Stress Test. B) Basal respiration measures oxygen consumption when no additional compounds are present. C) Maximum respiration is the highest level of oxygen consumption that a cell can achieve. D) ATP production by complex V is linked to the electron transport by complexes I-IV of the electron transport chain. E) Spare Respiratory Capacity indicates the degree to which the cell can increase its respiration in response to a bioenergetic demand. F) Non-mitochondrial respiration refers to oxygen consumption independent of oxidative phosphorylation. G) Production of mitochondrial ROS measurement with MitoSox fluorescence by flow cytometry. Values in the bar charts represent mean ± SEM. Comparisons were made using one-way ANOVA and Tukey's post hoc test. *$p < 0.05$ and ***$p < 0.001$ with respect to control; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ when compared to HbA1c < 7.

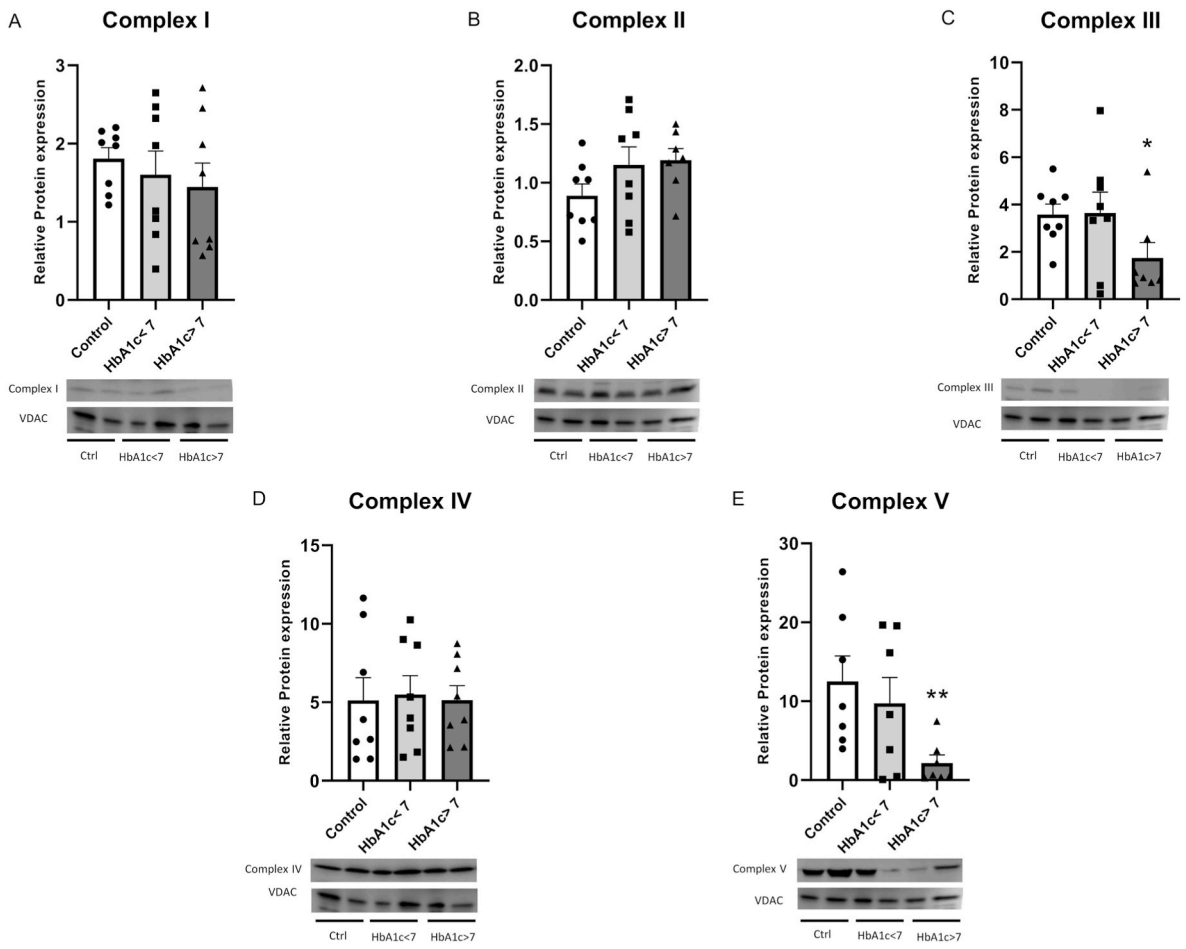

Fig. 2. OXPHOS protein levels of PBMCs from control individuals and subjects with poor and good glycaemic control of T2D. A) Complex I or NADH-dehydrogenase complex. B) Complex II or Ubiquinone or Coenzyme Q reductase. C) Complex III or Cytochrome *b-c*1 complex. D) Complex IV or Cytochrome-oxidase complex. E) Complex V or ATP synthase. Values in the bar charts represent mean ± SEM. Comparisons were made using one-way ANOVA and Tukey's post hoc test. *$p < 0.05$ and **$p < 0.01$ with respect to control.

can be observed by analysing neutrophil-endothelium interactions. In our study, we observed a significant decrease in the rolling velocity of neutrophils in both the T2D HbA1c < 7 and T2D HbA1c > 7 groups (Fig. 3A, $p < 0.001$) in comparison to controls. Concurrently, there was an increase in rolling flux and neutrophil adhesion in both the T2D HbA1c < 7 and T2D HbA1c > 7 groups (Fig. 3B and C, $p < 0.001$) in relation to controls. Additionally, there was an increase in rolling flux (Fig. 3B, $p < 0.001$) and neutrophil adhesion (Fig. 3C, $p < 0.001$) in the

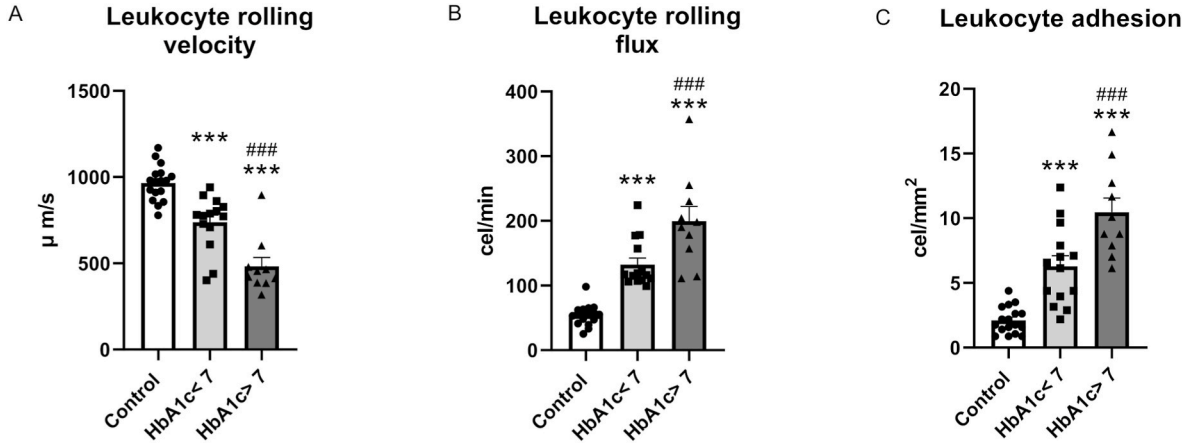

Fig. 3. Neutrophil-endothelium interaction parameters of control individuals and patients with poor and well glycaemic control of T2D. A) Neutrophil rolling velocity of neutrophils across the endothelial cell layer (µm/sec). B) Neutrophil rolling flux is the number, per unit of time, of neutrophils rolling across the endothelial cell layer (cells/min). C) Neutrophil adhesion is the number of neutrophils attached to the endothelium (cells/mm^2). Values in the bar charts represent mean ± SEM. Comparisons were made using one-way ANOVA and Tukey's post hoc test. ***$p < 0.001$ with respect to control; $^{###}p < 0.001$ with respect to HbA1c < 7.

T2D HbA1c > 7 versus T2D HbA1c < 7 group, along with a decrease in rolling velocity in the T2D HbA1c > 7 versus T2D HbA1c < 7 group (Fig. 3A, $p < 0.001$). These changes suggest that poor glycaemic control results in a greater level of neutrophil-endothelium interactions, which can accelerate the atherosclerotic process.

3.5. Levels of cytokines and adhesion molecules

We evaluated the inflammatory process in patients from different groups and failed to observe significant differences in IL-10 (Fig. 4A), IL-1β (Fig. 4B) and IL-6 (Fig. 4C), although IL-1β and IL-6 showed a tendency to increase in patients with T2D compared to controls ($p = 0.06$ and $p = 0.09$, respectively). Regarding TNF-α, an increase was observed in patients with T2D (both T2D HbA1c < 7 and T2D HbA1c > 7) compared to the control group (Fig. 4D, $p < 0.05$). Additionally, when adhesion molecule levels were measured, a significant increase in ICAM-1 (Fig. 4E, $p < 0.01$) and VCAM-1 (Fig. 3F, $p < 0.001$) was seen in the T2D HbA1c > 7 group with respect to controls. No differences were observed in terms of P-selectin (Fig. 4G). MPO levels in serum were also higher in the T2D HbA1c < 7 and T2D HbA1c > 7 groups than among controls (Fig. 4H, $p < 0.05$).

3.6. Correlation studies

To better understand the relationship between cellular OCR and glycaemic control of the disease, we explored correlations between the Seahorse data and HbA1c levels, observing a negative correlation of basal respiration ($r = -0.319$, $p < 0.05$), maximal respiration ($r = -0.350$, $p < 0.01$), and spare respiratory capacity ($r = -0.295$, $p < 0.05$) with blood HbA1c levels (Fig. 5). This leads us to believe that T2D patients with poor glycaemic control exhibit decreased mitochondrial oxygen consumption, pointing to dysfunction in the electron transport chain complexes and mitochondrial impairment.

An additional correlation study of neutrophils and MPO was performed, revealing a positive correlation between neutrophil count and MPO levels ($r = 0.388$, $p < 0.01$; Supplementary Fig. 3).

4. Discussion

This study highlights the importance of glycaemic control in T2D through the evaluation of endocrine and anthropometric parameters, OCR, mitochondrial ROS production, mitochondrial complex levels, neutrophil–endothelium interactions (rolling, rolling velocity and adhesion), pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), anti-inflammatory cytokines (IL-10), adhesion molecules (ICAM-1, VCAM-1, and P-selectin), and levels of MPO. In addition, we have analyzed the interrelationships of these parameters by performing correlation studies.

Chronic hyperglycaemia and hyperlipidaemia result in the overloading of the mitochondrial electron transport chain, which generally increases ROS production and impairs mitochondrial function. Our results show a significant impairment in the OCR of leukocytes from T2D patients with poor glycaemic control (T2D HbA1c > 7); specifically, basal OCR, maximal respiration and spare respiratory capacity were lower with respect to control subjects. Moreover, a significant decrease was observed in maximal respiration and spare respiratory capacity in the T2D HbA1c > 7 group compared to the T2D HbA1c < 7 group. A further notable finding was the significant increase in non-

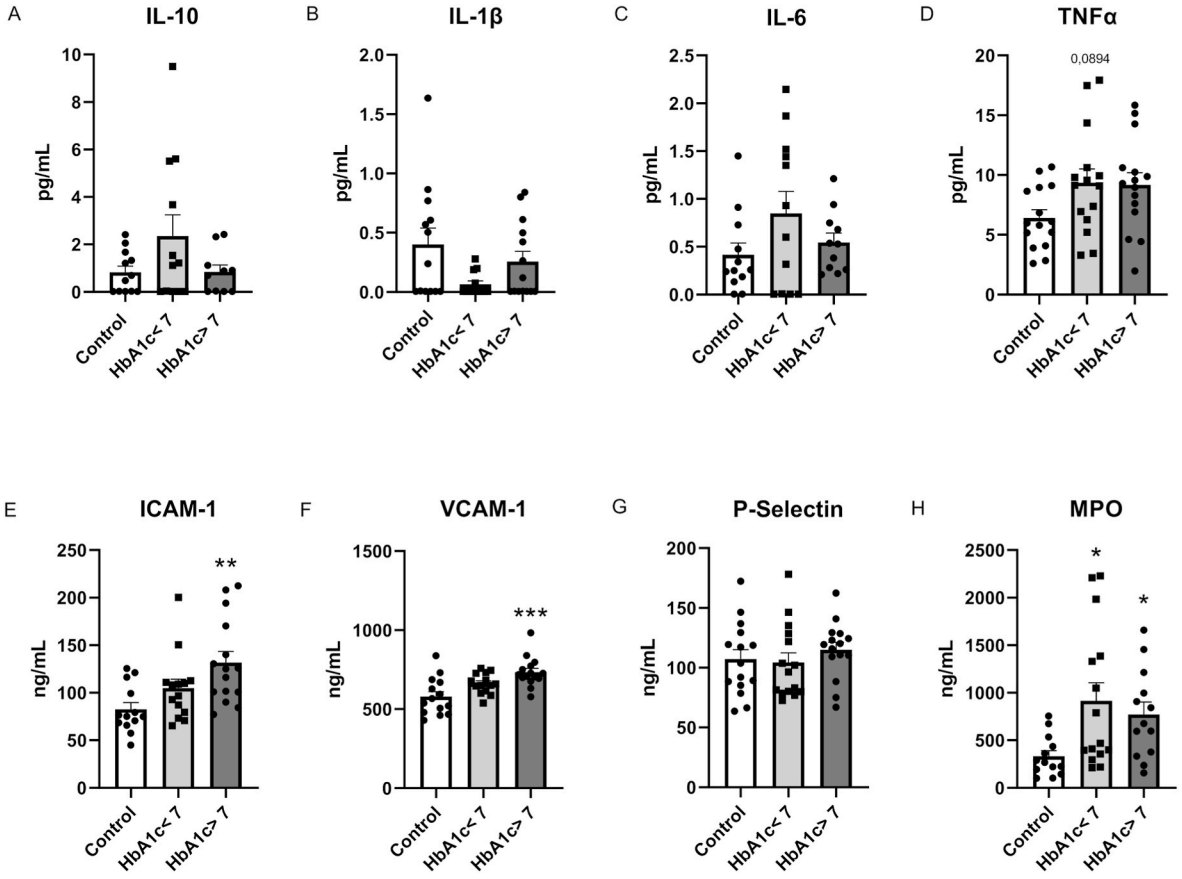

Fig. 4. Expression levels of inflammatory cytokines and inflammatory markers in serum of control individuals and patients with poor and good glycaemic control in T2D. A) Interleukin 10 (IL-10). B) Interleukin 1b (IL-1b). C) Interleukin 6 (IL-6). D) Tumor necrosis factor alpha (TNFα). E) Intracellular adhesion molecule 1 (ICAM-1). F) Vascular cell adhesion molecule 1 (VCAM-1). G) P-Selectin. H) Myeloperoxidase (MPO). Bars represent the means ± S.E.M. Comparisons were made using one-way ANOVA and Tukey's post hoc test. Asterisks indicate statistically significant differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with respect to control.

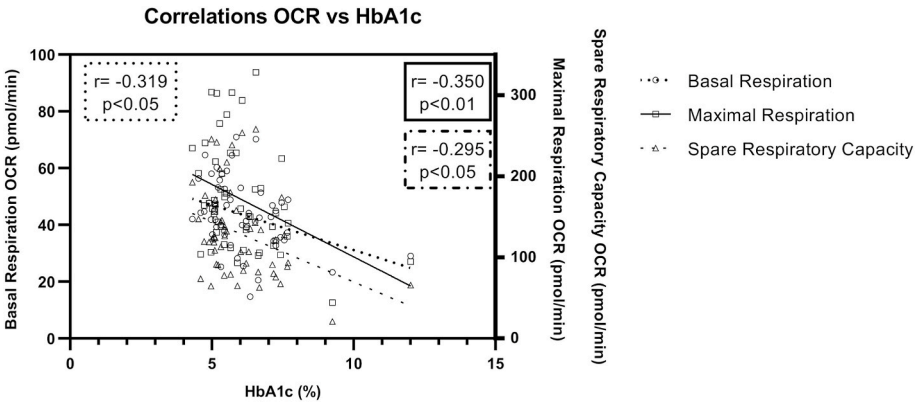

Fig. 5. Correlations between oxygen consumption and HbA1c level (%) in patients with T2D. Basal respiration, maximal respiration and respiratory reserve capacity show a negative correlation with the level of HbA1c. Spearman's correlation coefficient was used for correlation studies.

mitochondrial respiration in the T2D HbA1c < 7 groups compared to controls. In this respect, we appreciate that different blood cell types have different bioenergetic profiles [6]. Nevertheless, given that the number of PBMCs in each well was constant in our Seahorse experiment, and since the monocyte-to-lymphocyte ratio was similar among the groups, we suspect that the differences in bioenergetics that we observed were probably due to mitochondrial function. These findings indicate that patients with poor metabolic control exhibit reduced OCR, suggesting a decrease in the activity of OXPHOS complexes, which in turn is likely to lead to mitochondrial dysfunction.

Spare capacity estimates mitochondrial health and flexibility, indicating the cell's bioenergetic adaptation to pathological stress. Inadequate spare respiratory capacity is associated with neurological and cardiovascular chronic diseases [19] and the development of diabetic complications [20,21]. Furthermore, impaired spare respiratory capacity has been described *in vitro* in acute and chronic T2D in rodent heart and skeletal muscle cells [22]. In addition, it has been reported that PBMCs from diabetic patients with and without nephropathy do not differ with respect to basal and ATP-linked respiration, though a reduced maximal and spare respiratory capacity was observed in the study in question [21].

ETC integrity is a key factor for obtaining energy, and can be specially compromised in T2D development. In the present study, we have observed a decrease in mitochondrial complex III and complex V in T2D patients with poor glycaemic control versus controls, while differences were not found between patients with good glycaemic control and controls. In line with these results, T2D has been related to an impairment of mitochondrial complexes in subsarcolemmal mitochondria of T2D patients' right atrial tissue [23] and in animal models [24,25]. It is important to point out that a decrease in the subunits of these complexes might not reflect their true level of activity. In addition, electron transport chain proteins are physiologically expressed in excess of what is required to maintain OXPHOS [6]. Despite this, we observed an important decrease in Complexes III and V in the HbA1c group, which reflects how chronic hyperglycaemia affects mitochondrial homeostasis. Furthermore, although ATP production was not affected in our cohort of patients, our findings suggest that basal OCR, maximal OCR and spare capacity are compromised when HbA1c levels are above 7, which could in turn undermine the cell's ability to meet its energetic demands under conditions of stress. Determining supercomplex assembly and performing the Seahorse Mito Stress test upon stimulation with compounds that activate PBMCs [26] would seem to be effective strategies to better characterize how T2D and HbA1c impact on mitochondrial function and cellular bioenergetics in future studies.

In addition, we have seen enhanced mitochondrial ROS production in the PBMCs of T2D patients with respect to those of control subjects, an effect that was more evident in those whose glycaemic status was poorly controlled. These results suggest that glycaemic control plays a key role in the balance of mitochondrial complexes and ROS production. It is important to highlight that excessive levels of ROS can accelerate inflammatory pathways [27], while the accumulation of ROS can impair mitochondrial function due to the generation of mtDNA mutations and the accumulation of dysfunctional mitochondria [28]. Moreover, research in the field suggests that good glycaemic control reduces ROS production [29], in accordance with studies demonstrating high mitochondrial ROS production in T2D and relating it to the development of silent myocardial ischemia [30]. In this sense, it is important to underline that leukocytes are especially linked to ROS generation and are highly sensitive to the oxidative damage mediated by ROS [31]. Furthermore, oxidative stress induces the accumulation of dysfunctional mitochondria and is related to decreased insulin sensitivity and impaired β-cell insulin synthesis and secretion [32].

In general, inflammatory processes are associated with hypertension and atherosclerosis, and are therefore characterized by leukocyte recruitment to the arterial wall. In the present study, we have used an *in vitro* model in which human leukocytes flow over a monolayer of human endothelial cells with a shear stress similar to that observed *in vivo* [30]. This mimics the process that precedes inflammation *in vivo* (rolling and adhesion) and which is critical to homeostasis and vascular cell integrity. Our experimental system has been widely applied to visualize and analyze the multistep recruitment of leukocytes in these diseases, and allows the mechanisms of action implicated in this recruitment to be assessed. In this sense, it has been demonstrated that an inflammatory background favours the increase of leukocyte–endothelium interactions and promotes the early development of atherosclerotic events [33]. In the present study, we have observed that the presence of T2D enhanced rolling flux and adhesion and reduced the rolling velocity of neutrophils, an effect that was more evident in the group with HbA1c > 7 %, suggesting that glycaemic control plays an important role in the development of atherosclerosis. Indeed, several other studies have demonstrated the importance of leukocytes in the context of atherosclerosis [34,35]. In accordance with this idea, an increase in leukocyte–endothelium interactions has been related to oxidative stress in a human model of insulin resistance [36], while increased recruitment alongside impaired function of leukocytes has been reported during inflammation in a mouse model of T2D [37].

T2D is a chronic inflammatory state. In the present study we have observed a significant increase in TNF-α levels in both groups of T2D patients, while no significant differences were evident in terms of the proinflammatory cytokines IL-1β and IL-6 or the anti-inflammatory cytokine IL-10. It is possible that values would have been more noticeable if it were not for the hypolipemiant treatment received by most of T2D patients. In this sense, enhanced levels of TNF-α in leukocytes activated by ROS-induced oxidative stress are thought to impair glucose

uptake and inhibit insulin signaling [38].

Endothelial and immune cell activation can be assessed by measuring the soluble adhesion molecules VCAM-1, ICAM-1, and P-selectin. In this sense, it has been described that adhesion molecules are enhanced in patients with T2D [39]. In the present study, we show an increase in adhesion molecules, ICAM-1 and VCAM-1 in T2D patients with poor glycaemic control (T2D HbA1c > 7 % group). These results are compatible with a rise in the number of leukocyte–endothelium interactions, and it has been demonstrated that hyperglycaemia in both normal subjects and T2D patients can induce vasoconstriction, inflammation and an increase of adhesion molecules [40].

Finally, we observed an increase in MPO levels in both T2D groups. This pro-oxidant enzyme is mainly released by neutrophils as an antimicrobial response, but enhanced levels have been observed during chronic inflammatory situations and may trigger damage to the vascular walls [41]. We have previously demonstrated elevated levels of MPO in T2D patients, especially in those with vascular complications [42]. This increase in MPO levels might reflect higher numbers of neutrophils in T2D subjects.

To better understand the relationship between cellular OCR and glycaemic control of the disease, we performed correlations between the Seahorse data and HbA1c levels. Our findings revealed a negative low-to-moderate correlation of basal respiration, maximal respiration, and spare respiratory capacity with blood HbA1c levels, suggesting that glycaemic control is important for maintaining mitochondrial function. In this sense, poor glycaemic control of T2D patients (HbA1c > 7) would lead to a decrease in mitochondrial oxygen consumption and dysfunction in the electron transport chain complexes and mitochondrial impairment. In line with these results, an impairment of mitochondrial complexes has been reported in animal models with T2D [24] and subsarcolemmal mitochondria of T2D patients' right atrial tissue [43]. In fact, our group has previously highlighted the importance of glycaemic control for leukocyte homeostasis, demonstrating that T2D-related alterations in mitochondrial dynamics, the NLRP3 inflammasome and endoplasmic reticulum stress are more pronounced in T2D patients with poor glycaemic control of the disease [44–46].

Our study has some limitations, including the higher number of women participants in the control group and the greater age and BMI of T2D subjects. That said, we have tried to minimize the impact of these differences by adjusting the statistical analyses for age, sex and BMI.

Considered together, our findings highlight the key role that glycaemic control plays in mitochondrial oxygen consumption, mitochondrial complex levels, mitochondrial ROS production, neutrophil-endothelium interactions, adhesion molecules and inflammatory markers in T2D. These results highlight the importance of ensuring and maintaining adequate glycaemic control for preventing mitochondrial dysfunction and limiting the risk of developing cardiovascular diseases associated with T2D.

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CRediT authorship contribution statement

Julia Cacace: Writing – original draft, Investigation, Formal analysis, Data curation. **Clara Luna-Marco:** Methodology, Investigation, Data curation. **Alberto Hermo-Argibay:** Methodology, Investigation. **Catherine Pesantes-Somogyi:** Investigation, Formal analysis. **Omar A. Hernández-López:** Visualization, Investigation. **María Pelechá-Salvador:** Investigation, Data curation. **Celia Bañuls:** Resources, Formal analysis. **Nadezda Apostolova:** Validation, Investigation. **Luis de Miguel-Rodríguez:** Visualization, Investigation. **Carlos Morillas:** Supervision, Resources. **Milagros Rocha:** Writing – review & editing, Supervision, Resources, Conceptualization. **Susana Rovira-Llopis:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Víctor M. Víctor:** Writing – review & editing, Writing – original draft, Resources, Conceptualization.

Declaration of competing interest

None.

There is no financial/personal interest or belief that could affect the objectivity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2025.103516.

Data availability

Data will be made available on request.

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