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The reduction of NDUFC2 expression is associated with mitochondrial impairment in circulating mononuclear cells of patients with acute coronary syndrome

Salvatore Raffa [a,b,1,2], Xiao Lan Diana Chin [c,1,2], Rosita Stanzione [d,1], Maurizio Forte [d,1], Franca Bianchi [d,1], Maria Cotugno [d,1], Simona Marchitti [d,1], Andrea Micaloni [b,1], Giovanna Gallo [a,1], Leonardo Schirone [e,1], Giuliano Tocci [a,d,1], Roberto Violini [c,1], Maria Rosaria Torrisi [a,b,1], Massimo Volpe [a,d,1], Speranza Rubattu [a,d,*,1]

[a] Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University of Rome, Italy
[b] Ultrastructural Pathology Lab., Medical Genetics and Advanced Cellular Diagnostics Unit, Sant'Andrea University Hospital, Rome, Italy
[c] Cardiology Unit, S. Camillo-Forlanini Hospital, Rome, Italy
[d] I.R.C.C.S. Neuromed, Pozzilli, Isernia, Italy
[e] Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy

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ABSTRACT

Background: Deficiency of NADH dehydrogenase [ubiquinone], the mitochondrial complex I, represents an emerging mechanism of cardiovascular diseases. Ndufc2, a subunit of mitochondrial complex I, is involved in stroke development. We aimed to gain some insights on the role of Ndufc2 into acute coronary syndrome (ACS) through the assessment of its gene expression, along with that of anti-oxidant proteins and of mitochondrial function parameters, in circulating mononuclear cells (PBMCs) of ACS versus stable angina (SA) patients. The impact of NDUFC2 silencing in human endothelial and vascular smooth muscle cells was assessed in vitro.
Methods and results: One hundred twenty-three patients presenting with SA ($n = 41$) or ACS ($n = 82$) were enrolled. PBMCs were used to assess the gene expression level of: NDUFC2, uncoupling protein 2 (UCP2), superoxide dysmutases 1 and 2 (SOD1, SOD2), levels of ROS and ATP. The mitochondrial dysfunction was assessed by cytofluorimetry; the structural damage by transmission electron microscopy. Cell viability, angiogenesis, markers of atherogenesis were evaluated in NDUFC2-silenced vascular cells.
NDUFC2 mRNA level was significantly downregulated, along with UCP2, SOD1, SOD2 expression, in ACS patients. We found significant increases of ROS levels, reduced ATP levels, higher degree of mitochondrial structural damage and dysfunction in ACS patients. In vitro, NDUFC2 silencing favored mechanisms involved in atherogenesis and plaque vulnerability.
Conclusions: A significant reduction of NDUFC2 expression is detected in ACS. In vitro, NDUFC2 silencing affects vascular cell viability and angiogenesis while stimulating the expression of markers of plaque rupture. Our observations suggest that these mechanisms may contribute to ACS development.

Abbreviations: ACS, acute coronary syndrome; CAD, coronary artery disease; CD40L, CD40 ligand; CVD, cardiovascular diseases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HASMC, human aortic smooth muscle cell; HUVEC, human humbilcal vein endothelial cell; ICAM, intercellular adhesion molecule 1; IMM, inner mitochondrial membrane; MMP9, matrix metallopeptidase 9; Ndufc2, NADH dehydrogenase [ubiquinone] 1 subunit; NO, Nitric Oxide; NSTEMI, non-ST elevation myocardial infarction; OMM, outer mitochondrial membrane; PBMCs, circulating mononuclear cells; ROS, reactive oxygen species; RT-PCR, Real-time quantitative polymerase chain reaction; SA, stable angina; SOD, superoxide dysmutases; STEMI, ST elevation myocardial infarction; TEM, Transmission Electron Microscope; TNFα, tumor necrosis factor α; UCP2, uncoupling protein 2; VCAM, vascular adhesion molecule 1.

* Corresponding author at: Clinical and Molecular Medicine Department, School of Medicine and Psychology, Sapienza University, Sant'Andrea Hospital, Rome, IRCCS Neuromed, Pozzilli, Italy.
E-mail address: rubattu.speranza@neuromed.it (S. Rubattu).

[1] These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
[2] These authors equally contributed to the work.

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1. Introduction

Among cardiovascular diseases (CVD) coronary artery disease (CAD) still remains the most common cause of death worldwide [1–3]. CAD can clinically manifest in both stable and unstable forms, such as stable angina (SA) and acute coronary syndrome (ACS). The abrupt clinical presentation of ACS is a strong signal of discontinuity in the natural history of atherothrombosis. Despite the identification of many risk factors involved in CAD and the identification of several mechanisms involved in triggering the acute inflammatory process, the specific factors that contribute to plaque instability still remain poorly understood [4–7]. The accumulation of reactive oxygen species (ROS), a mechanism underlying several CVDs, can play a major role.

Of note, mitochondria are the primary intracellular source of ROS, as they generate huge numbers of oxidative-reduction reactions and use large amounts of oxygen [8]. The balance between mitochondrial redox reactions with antioxidant defense becomes essential to maintain normal mitochondrial function and preserve cell viability [8]. Under physiologic conditions, several tightly controlled oxidative pathways contribute to ROS production, while several intra and extracellular antioxidant enzymatic mechanisms account for ROS elimination, including manganese superoxide dysmutases (SOD) and uncoupling proteins (UCPs) [9–11]. Importantly, previous studies underscored the role of mitochondrial dysfunction in the development of atherosclerosis [12].

A contributory role of mitochondrial dysfunction dependent from a complex I deficiency, that is frequently encountered in human diseases, has not been fully elucidated yet in CAD, although some evidence exists in this regard [13]. Recently, the Ndufc2 (NADH dehydrogenase [ubiquinone] 1 subunit) has emerged as a key fundamental subunit of mitochondrial complex I that is needed for the appropriate assembly and activity of the complex. Its deficiency causes complex I dysfunction and severe mitochondrial impairment, and it behaves as a key pathogenetic mechanism for stroke in both a rat model and in humans [14,15].

We presently tested the hypothesis that Ndufc2 deficiency may be detected in ACS, as a potential contributory pathogenetic mechanism. For this purpose, we aimed at comparing the expression pattern of NDUFC2 expression in ACS versus SA patients. Furthermore, we aimed at characterizing anti-oxidant mechanisms, such as UCP2, SOD1 and SOD2, in both groups of patients. We related these expression patterns with the degree of mitochondrial dysfunction and ultrastructural damage, and with the levels of both oxidative stress and ATP in these types of patients. Furthermore, we explored the pathological implications of NDUFC2 downregulation in vascular cells to better support the human evidence.

2. Materials and methods

2.1. Human study design

Patients presenting with SA and ACS who underwent coronary angiography were consecutively enrolled. In all patients cardiovascular risk factors were carefully examined. Medications taken on admission were recorded. Furthermore, in order to compare genes expression, levels of ROS, ATP, mitochondrial membrane potential and ultrastructural quantitation of mitochondrial damage in peripheral blood mononuclear cells (PBMCs) of SA and ACS patients, a group of 10 age and sex-matched healthy volunteer subjects was recruited. A peripheral venous blood sample (15 ml) was drawn from each patient and control subject for the PBMCs isolation.

The study protocol conformed to the ethical guidelines of the 1975 declaration of Helsinki and it was approved by the hospital's ethics committees. All patients gave their informed written consent before entering the study.

2.2. In vitro study design

Human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) were used as vascular models in order to test the effects of NDUFC2 downregulation.

Experiments of cell viability, angiogenesis and gene expression of markers of atherogenesis were performed in NDUFC2-silenced vascular cells and compared to not silenced cells.

2.3. Statistical analysis

All data were entered into Microsoft Office for Windows. All calculations were generated using SPSS, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Statistical significance was stated at the $p < 0.05$ value. The GraphPad Prism (V. 5.01 GraphPad Software, Inc., La Jolla, CA, USA) statistical software was used for the statistical analysis.

An expanded Materials and Methods section is available in the Supplementary file.

3. Results

3.1. Patient characteristics

Out of 152 screened patients, 123 subjects (86 males with a median age of 72 years; 37 females with a median age of 67 years) were enrolled into the study. The remaining patients were not included for the following reasons: worsening heart failure ($n = 12$), current infections ($n = 9$), lack of consent to participate to the study ($n = 8$). Out of the 123 subjects, 82 patients (66.7%) had a diagnosis of ACS, while 41 patients (33.3%) presented with SA. Baseline characteristics of the two study groups are illustrated in Supplementary Table 1. Briefly, the two groups were similar for age and sex. Current smoking habit was more frequent among ACS patients. Patients with SA received more frequently statin therapy as compared to ACS patients. Levels of CRP were higher in ACS. Among angiographic parameters of CAD burden, ACS patients had significantly higher critical stenosis, defined as >70% stenosis, whereas SA patients had a higher number of subcritical stenoses (defined as <70% stenosis). SA patients tended to have a higher number of previous percutaneous coronary interventions (PCI).

3.2. PBMCs gene expression

The analysis of NDUFC2 mRNA expression level showed a significantly reduced expression in patients with ACS compared to both SA patients and controls ($P < 0.0001$; Fig. 1A). A downregulation of UCP2 was observed in ACS vs both SA and control groups ($p < 0.001$; Fig. 1B). A lower expression of SOD1 and SOD2 compared to the control group ($p < 0.0001$; Fig. 1C–D) was present in ACS patients. Of note, SOD2 expression was significantly upregulated in SA compared to ACS patients ($p < 0.0001$; Fig. 1D).

3.3. Mitochondrial function and ROS levels assessment

In order to study the mitochondrial function, mitochondrial membrane potential, ATP levels and ROS production were assessed in ACS_PBMCs with respect to SA_ and CTRL_PMBCs.

The JC-1 staining index (red/green ratio; Fig. 2A), the percentage of cells with a high mitochondrial membrane potential (JC-1 aggregate Fig. 2B) and the $\Delta\psi m$ values (TMRM staining; Fig. 2C) were significantly lower in ACS patients with respect to SA and CTRL subjects ($p < 0.05$). In parallel, the levels of ATP were also markedly reduced in ACS patients ($p < 0.0001$ vs CTRL, Fig. 2D).

To assess whether the mitochondrial dysfunction observed in the ACS_PBMCs could be related to the generation of increased oxidative stress, we evaluated the intracellular and mitochondrial ROS levels by cytofluorimetric analysis. Levels of cytoplasmic ROS were significantly increased in both groups of patients, and particularly in ACS patients compared to control (Fig. 2E; $p < 0.0001$); instead, mitochondrial ROS were higher only in ACS patients (Fig. 2F; $p < 0.05$).

3.4. Assessment of mitochondrial structural damage

Based on the above-described functional data, we wanted to quantify the morphological alterations in mitochondria. The distribution of Mt-G categories and the burden of overall damage were similar among PBMCs from CTRL and SA patients (64% and 68%; $p <$ NS; Fig. 3A and C–D); in contrast, PBMCs from ACS patients showed an overall damage that was significantly higher (85%; $p < 0.01$; Fig. 3A and E–F).

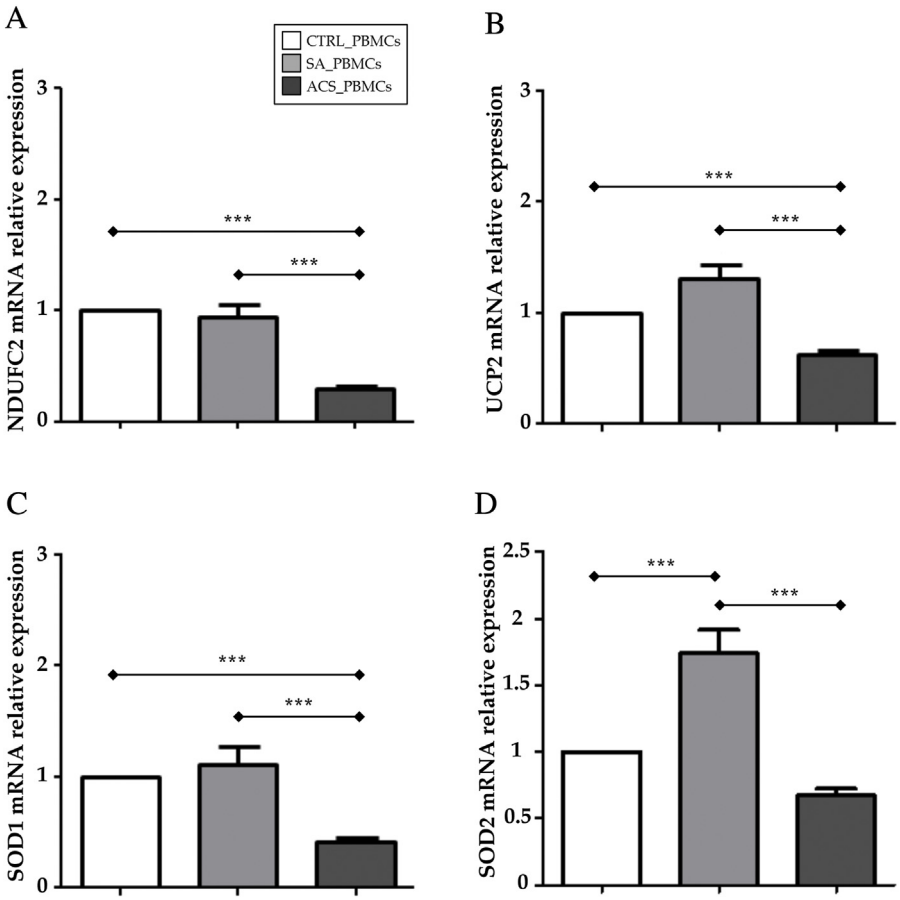

Fig. 1. Differential expression of NDUFC2 and antioxidant proteins in PMBCs from ACS vs SA patients. (A). Relative expression of NDUFC2 mRNA levels from PBMCs from control subjects (Ctrl), ACS and SA patients. ACS patients showed significantly lower amount of NDUFC2 mRNA expression as compared to both control (*** = $p < 0.0001$) and SA patients (*** = $p < 0.0001$). **(B)**. Relative expression of UCP2 mRNA levels from PBMCs of the same groups. ACS patients showed significantly lower levels of UCP2 mRNA as compared to both control (*** = $p < 0.0001$) and SA patients (*** = $p < 0.0001$). SA patients tended to upregulate UCP2 compared to the control group ($p < 0.001$). **(C–D)**. Relative expression of SOD1 (panel C) and SOD2 (panel D) mRNA levels from PBMCs of the same groups. ACS patients showed a significant downregulation of SOD1 and SOD2 mRNA expression as compared to both control (*** = $p < 0.0001$) and SA patients (*** = $p < 0.0001$). Of note, SOD2 was significantly upregulated in SA patients as compared to the control group (*** = $p < 0.0001$).

These findings were corroborated by the IMM/OMM index analysis, which was characterized by the values of 1.90 ± 0.12 and 1.78 ± 0.04 for PBMCs from SA and ACS subjects respectively ($p < 0.05$; Fig. 3B).

3.5. In vitro studies

Fig. 4 shows the results of the in vitro studies performed to test the impact of NDUFC2 downregulation in both HUVECs and HASMCs. First, the efficiency of gene silencing was confirmed by RTPCR (NDUFC2 expression, CTR: 1 ± 0.14, siRNA: 0.04 ± 0.017 in HUVEC, $p < 0.01$; CTR: 1 ± 0.13, siRNA: 0.012 ± 0.01 in HASMC, $p < 0.01$). We observed a significant reduction of cell viability and a significant increase of cell necrosis in NDUFC2-silenced HUVECs (Fig. 4A, B). Of interest, NDUFC2 knockout severely affected angiogenesis in HUVECs (Fig. 4C). Similarly, NDUFC2-silenced HASMC displayed a reduction of live cells and an increase of apoptotic cells (Fig. 4D, E). We also studied the expression of genes involved in inflammation, adhesion and plaque instability, that represent key processes involved in atherogenesis [16,17]. As shown in Fig. 4F, the expression of tumor necrosis factor α (TNFα), a marker of inflammation, increased in NDFUC2-silenced HUVECs. In parallel, expression of intercellular adhesion molecule 1 (ICAM) and vascular cell adhesion molecule 1 (VCAM), two surface adhesion molecules, was significantly higher in knockout cells. We also observed increased levels of TNFα in NDUFC2-silenced HASMCs (Fig. 4G). Notably, the expression of matrix metallopeptidase 9 (MMP9) and CD40 ligand (CD40L), two markers of plaque instability [18,19], were significantly higher in silenced HASMCs compared to control cells (Fig. 4G).

4. Discussion

In the present study we aimed at characterizing the gene expression of Ndufc2, a key subunit of mitochondrial complex I, along with ROS generation, expression of antioxidant proteins and parameters of mitochondrial function and structure in the attempt to gain novel insights on mitochondrial dysfunction as a potential contributor to ACS development. The original result of our study was that the relative mRNA expression of Ndufc2 and of the antioxidant proteins was significantly impaired in patients with ACS compared to both SA patients and controls. This evidence was associated with reduction of mitochondrial membrane potential and ATP synthesis and with overproduction of ROS, all signs of mitochondrial dysfunction. A set of in vitro studies confirmed that NDUFC2 downregulation significantly affected vascular health and physiology, enhancing the mechanisms responsible for atherogenesis and plaque instability.

The choice of the cell type used in the clinical investigation was based on the evidence that circulating mononuclear cells closely reflect the presence and progression of CAD; in fact, this cell type has been previously used to monitor CAD [20]. Moreover, we recently characterized the mitochondrial dysfunction in circulating mononuclear cells as a marker of chronic heart failure [21].

Mitochondria represent the major source of energy for cells in the form of ATP via electron transfer through multimeric complexes and they are also the primary intracellular source of ROS [8]. Increasing evidence suggests that accumulation of ROS, as a result of an imbalance between ROS production and clearance, leads to mitochondrial protein

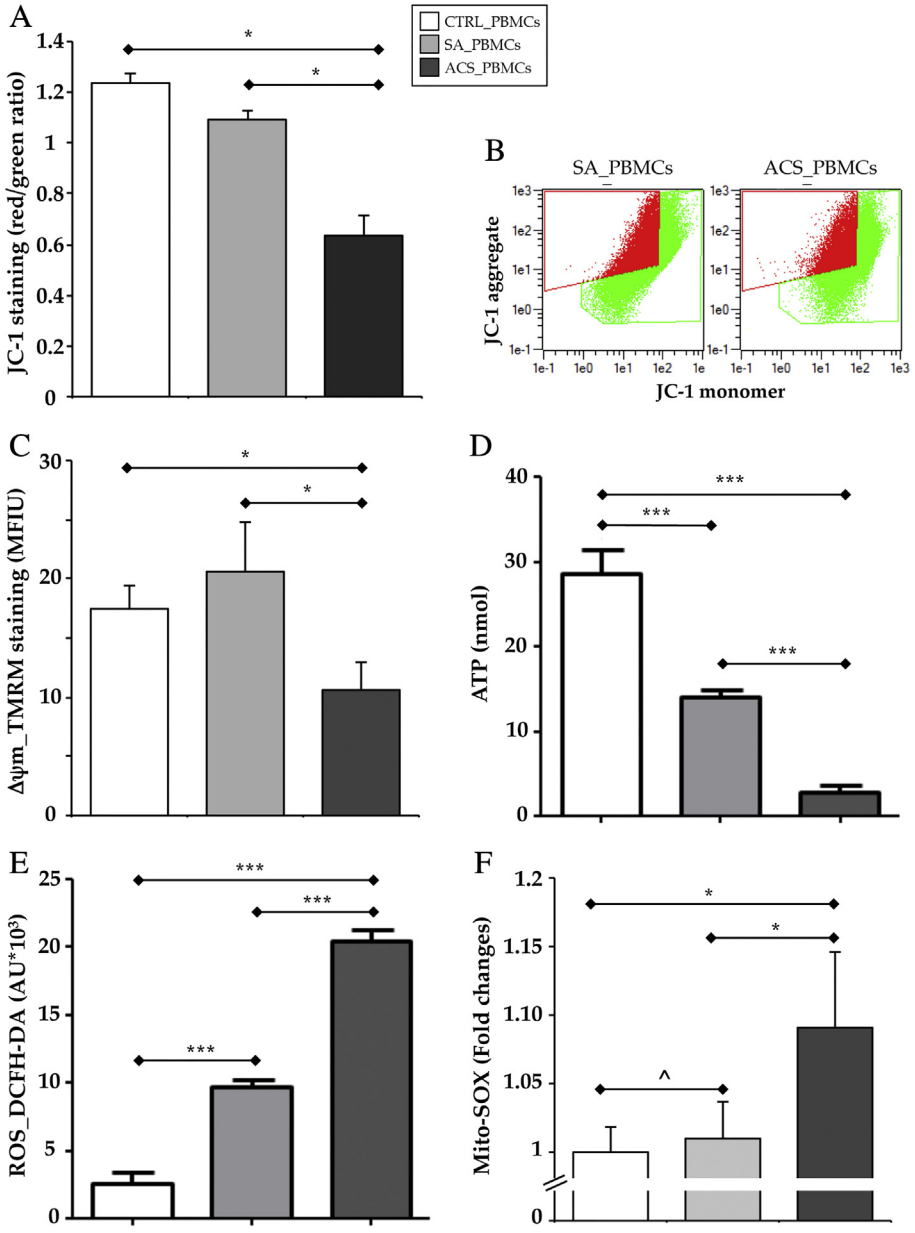

Fig. 2. Mitochondrial function and ROS levels assessment in PBMCs from ACS vs SA patients. (A–C). JC-1 and TMRM cytofluorimeric assay for mitochondrial membrane potential (Δψm). The PBMCs from ACS patients showed a significant reduction of mitochondrial potential membrane respect to PBMCs from both SA patients and control subjects (panels A–C; $^{*}p < 0.05$). **(D).** The ATP production was lower in ACS patients respect to both SA patients and control subjects (panel D; $^{***}p < 0.0001$). **(E–F).** Cytofluorimetric assay of cytoplasmic (E) and mitochondrial ROS (F) generation. The cytoplasmic and mitochondrial ROS levels were higher in ACS respect to both SA patients and control subjects ($^{*}p < 0.05$; $^{***}p < 0.0001$; $^{\wedge}p$ = NS).

damage with subsequent alteration of mitochondrial function and further increase of ROS generation. Inflammation and oxidative stress are interrelated and they form a vicious cycle during the progression of atherosclerosis, plaque rupture and atherothrombosis [22].

Ndufc2 is a subunit of complex I, known as NADH:ubiquinone oxidoreductase. The latter is responsible of about 40% of the proton motive force that drives ATP synthesis. Downregulation of Ndufc2 results in an impairment of complex I assembly and hence reduced complex I activity with decreased ATP synthesis and increased ROS generation [15]. Recently, we found that Ndufc2 gene inhibition led to deficient complex I assembly, mitochondrial dysfunction and increased stroke susceptibility in the animal model of SHRSP with subsequent translation of findings to the human disease [14]. NDUFC2 has also been shown to be downregulated in skeletal muscle cells of subjects affected by insulin resistance and type II diabetes mellitus [23]. Bendavia, a mitochondria-targeting tetrapeptide (D-ArgdimethylTyr-Lys-Phe-NH2), has been reported to protect coronary artery reperfusion in animal models of occlusion/reperfusion injury after myocardial infarction [24]. Moreover, chronic administration of Bendavia in rats after acute myocardial infarction reduced ROS production, improved cardiac function and reduced remodeling by maintaining normal mitochondrial structure and energy-producing ability, as well as protecting cardiac myocytes from oxidative stress [25]. These findings were associated with Ndufc2 gene stimulation at the infarct border zone [25]. No evidence has been reported so far in human myocardial infarction with regard to Ndufc2 modulation. Our findings suggest a potential contributory role of Ndufc2 downregulation into the development of ACS in humans.

Among important physiological regulators of mitochondrial ROS production is the uncoupling proteins family [26]. The activation of these inner mitochondrial membrane anion transporters allows protons to leak back into the mitochondrial matrix, decreasing the mitochondrial membrane potential and counteracting ROS generation [27]. The

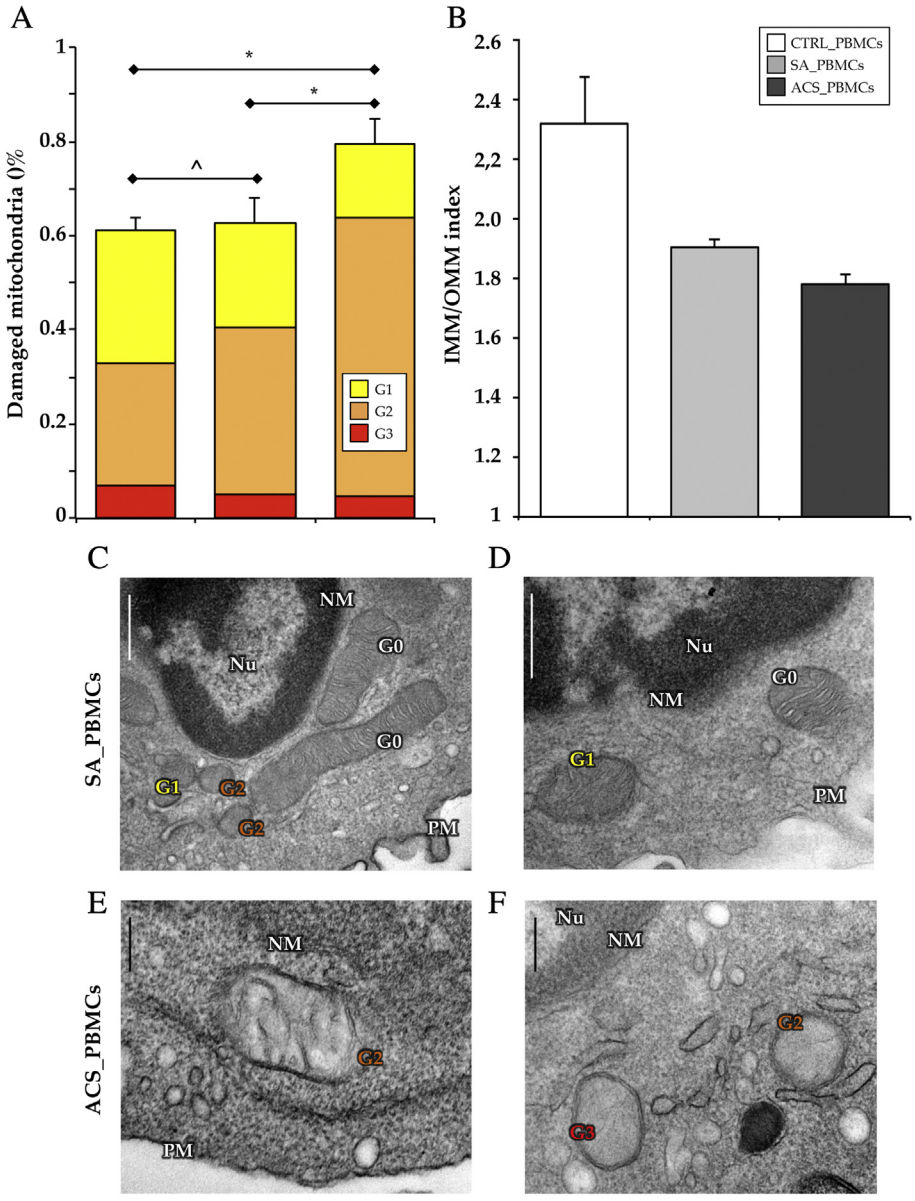

Fig. 3. Quantitative analysis of ultrastructural damage in mitochondria from PBMCs. (A). Graphical representation of the ultrastructural Mt-grading of damage. The burden of overall damage was higher in ACS_PBMCs with respect to SA_ and CTRL_PBMCs (*$p < 0.05$). **(B).** Graphical representation of the IMM/OMM values. By applying the IMM/OMM index associated with convolution loss of inner mitochondrial membrane, we observed a significantly lower index values in the ACS_PBMCs compared to SA_(*$p < 0.05$) and CTRL_PBMCs (**$p < 0.01$). **(C–F).** Representative micrographs of ultrastructural damage in mitochondria from SA_ and ACS_PBMCs. The burden of mitochondrial damage was higher particularly in ACS_PBMCs (E–F); the mitochondria were characterized by degeneration of convolutions related to lack of the inner membrane and subsequently by reduction of mitochondrial area with intact cristae. The PBMCs obtained from SA_PBMCs showed mitochondria with normal morphology or only with slight damage after stimulation (C–D). (TEM micrographs, uranyl acetate/lead citrate; Legend: NM, nuclear membrane, PM, plasma membrane; Nu: Nucleus; Gx: grade of mitochondrial damage).

regulatory role of UCP2 in atherogenesis has been previously demonstrated from observations where transplantation of bone marrow from UCP2-deficient mice to LDL receptor-deficient mice markedly increased atherosclerotic lesion size and increased nitrotyrosine staining in plaques [28]. The lack of UCP2 in blood cells accelerated atherosclerotic plaque development and induced macrophage-rich but collagen poor plaque phenotype, which supports a protective role for blood cell-derived UCP2 in the early stages of atherosclerosis [28]. Our current findings suggest that patients with a significant downregulation of UCP2 may be more prone to develop plaque instability and rupture, whereas normal or overexpression of UCP2 can be cardioprotective.

Antioxidant enzymes, such as SOD1 and SOD2, play an important scavenging role in reducing the harmful effects of excess mitochondrial ROS generation. SOD2 (also known as manganese SOD) catalyzes the dismutation of O_2^- to H_2O_2 in the matrix whereas SOD1 (also known as Cu/ZnSOD) does it in the intermembrane space. Cho et al. demonstrated that decreased SOD2 expression increased mitochondrial ROS in coronary endothelial cells from type II diabetic mice [29]. The increase in ROS generation led to a reduction of Nitric Oxide (NO) bioavailability, which is the hallmark of endothelial dysfunction. This in turn caused an attenuated vascular relaxation and atherosclerosis followed by a decrease in coronary blood flow, and hence an increase in myocardial ischemia. Overexpression of SOD2 has been reported by Chen et al. to offer protection against ischemia/reperfusion injury. In fact, SOD2 overexpression alone has been shown to reduce infarct size by 35% in vivo [30]. On the other hand, heterozygous deficiency of SOD2 impaired post-ischemic recovery of the myocardium in mice [31]. Whereas the cardioprotective role of SOD2 is well established, little is known about the role of SOD1 in CAD. Yoshida et al. demonstrated that SOD1 knockout mouse hearts were more susceptible to ischemic reperfusion injury

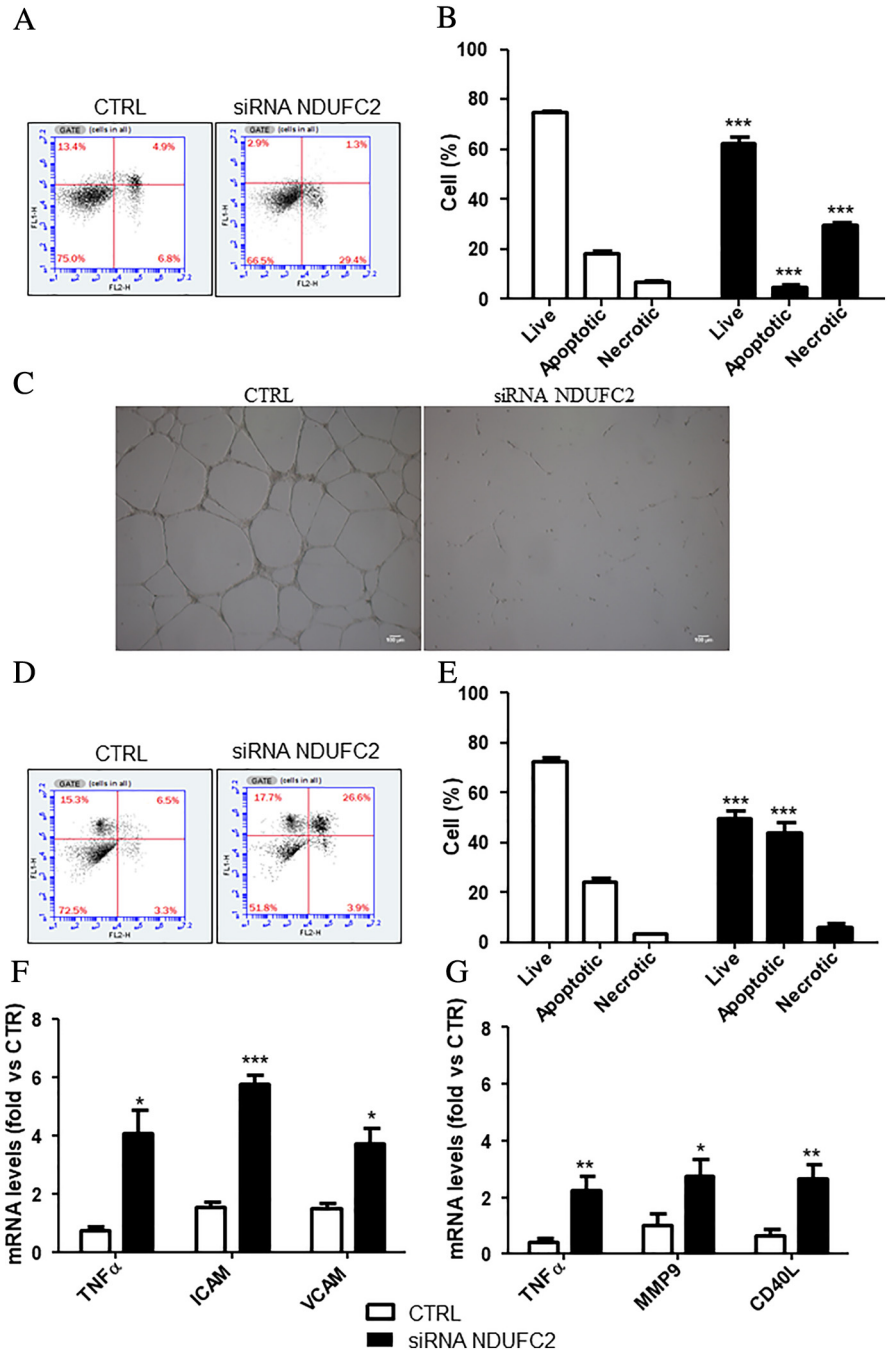

Fig. 4. Impact of NDUFC2 downregulation in human endothelial (HUVEC) and vascular smooth muscle (HASMC) cells in vitro. (A–B). FACS analysis of cell viability, apoptosis and necrosis in HUVEC. Representative dot plots **(A)** and corresponding percent values **(B)**. **(C).** Representative images of matrigel assay in HUVEC silenced or not for NDUFC2. **(D–E).** FACS analysis of cell viability, apoptosis and necrosis in HASMC. Representative dot plots **(D)** and corresponding percent values **(E)**. **(F).** Expression level, as detected by RTPCR, of markers of inflammation (TNFα) and adhesion (ICAM, VCAM) in HUVEC silenced or not for NDUFC2. **(G).** Expression level, as detected by RTPCR, of markers of inflammation (TNFα) and plaque instability (MMP9, CD40L) in HASMC silenced or not for NDUFC2. CTRL indicates control cells (no target siRNA) whereas siRNA-NDUFC2 indicates cells silenced for NDUFC2. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ siRNA-NDUFC2 vs CTRL cells.

compared with corresponding wild-type mouse hearts, suggesting that the SOD1 gene constitutes an important defense element for the hearts [32]. Our findings demonstrate that mitochondrial antioxidant mechanisms are compromised in ACS patients, likely favoring excess ROS generation, increased inflammation and plaque instability. Furthermore, the observation that SOD2 increases in SA patients, consistently with previous findings [33], may suggest that it plays a protective role in this group of patients by possibly preventing plaque instability.

Consistently with our previous data, we found that dysfunctional mitochondria produced less ATP, particularly in ACS patients. We also detected higher levels of ROS in ACS. These observations were supported by the high degree of mitochondrial structural damage detected in ACS patients. In fact, the mitochondrial membrane potential was decreased and the ultrastructural damage, as documented by the IMM/OMM ratio, was greatly increased in ACS. The observed differences in smoking habit and statins use between SA and ACS patients may have contributed to the results of the present study.

Of note, the findings obtained through the in vitro studies provided a major support to the human evidence by showing that NDUFC2 downregulation in vascular cells promotes deleterious effects on cell viability, impairs angiogenesis, stimulates the release of molecules involved in atherogenesis and plaque instability.

Thus, the NDUFC2-dependent mitochondrial dysfunction appears as a relevant deleterious contributory mechanism to atherogenesis and plaque rupture.

4.1. Study limitations

As a limitation of the human study, we detected only genes and not proteins expression due to technical difficulties in achieving sufficient amount of protein samples from PBMCs.

5. Conclusions

Our clinical investigation has shown that a significant reduction of NDUFC2 expression, which is known to result in altered complex I assembly and activity, is present in PBMCs of ACS patients. In vitro, the NDUFC2 downregulation severely affects vascular cell viability, impairs the angiogenic properties of endothelial cells and favors vascular inflammation, cell adhesion and plaque vulnerability.

Moreover, the antioxidant mechanisms are compromised during ACS with consequent reduced mitochondrial membrane potential, reduced ATP synthesis, ROS overproduction and increased damage of mitochondrial structure.

Our results support the notion that oxidative damage of mitochondrial proteins leads to progressive dysfunction and that mitochondrial dysfunction is a mechanism involved in the pathogenesis of several CVDs including ACS.

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Conflicts of interest

There are no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.02.027.

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