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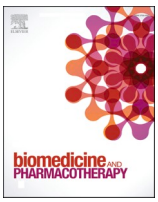

Apolipoprotein J protects cardiomyocytes from lipid-mediated inflammation and cytotoxicity induced by the epicardial adipose tissue of diabetic patients

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ABSTRACT

Diabetic patients present increased volume and functional alterations in epicardial adipose tissue (EAT). We aimed to analyze EAT from type 2 diabetic patients and the inflammatory and cytotoxic effects induced on cardiomyocytes. Furthermore, we analyzed the cardioprotective role of apolipoprotein J (apoJ).

EAT explants were obtained from nondiabetic patients (ND), diabetic patients without coronary disease (DM), and DM patients with coronary disease (DM-C) after heart surgery. Morphological characteristics and gene expression were evaluated. Explants were cultured for 24 h and the content of nonesterified fatty acids (NEFA) and sphingolipid species in secretomes was evaluated by lipidomic analysis. Afterwards, secretomes were added to AC16 human cardiomyocytes for 24 h in the presence or absence of cardioprotective molecules (apoJ and HDL). Cytokine release and apoptosis/necrosis were assessed by ELISA and flow cytometry.

The EAT from the diabetic samples showed altered expression of genes related to lipid accumulation, insulin resistance, and inflammation. The secretomes from the DM samples presented an increased ratio of pro/anti-atherogenic ceramide (Cer) species, while those from DM-C contained the highest concentration of saturated NEFA. DM and DM-C secretomes promoted inflammation and cytotoxicity on AC16 cardiomyocytes. Exogenous Cer16:0, Cer24:1, and palmitic acid reproduced deleterious effects in AC16 cells. These effects were attenuated by exogenous apoJ.

Diabetic secretomes promoted inflammation and cytotoxicity in cardiomyocytes. This effect was exacerbated in the secretomes of the DM-C samples. The increased content of specific NEFA and ceramide species seems to play a key role in inducing such deleterious effects, which are attenuated by apoJ.

1. Introduction

Diabetic patients are at high risk for coronary artery disease (CAD) and heart failure (HF). The presence of major cardiovascular (CV) risk factors and interrelated metabolic and inflammatory alterations is involved in the development and progression of CAD in diabetes. HF can be commonly caused by CAD, although it can also occur in the absence of CAD in diabetic patients, which is known as diabetic cardiomyopathy [1]. Several mechanisms have been involved in the pathogenesis of this disease, such as lipid oxidation, intramyocardial triglyceride accumulation, and impaired glucose utilization. Overall, these alterations eventually lead to enhanced inflammatory response and the apoptosis of cardiomyocytes [1].

Epicardial adipose tissue (EAT) is the visceral fat depot over the heart

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distributed in atrioventricular and interventricular grooves, coronary arteries, and myocardium [2]. EAT works not only as a simple fat storage depot but also as an extremely active organ that interacts with surrounding cells. On this basis, EAT is more similar to visceral than to subcutaneous adipose tissue [2]. The connection between EAT and neighboring structures is mediated through the release of lipid species, cytokines, adipokines, and chemokines from adipocytes or infiltrated macrophages. In CAD patients, EAT has been described to show higher sized adipocytes and increased macrophage infiltration [3,4] and to strongly induce the release of inflammatory molecules [5–9], thereby contributing to the development of coronary disease and myocardial dysfunction [10,11]. In diabetic patients, the presence of CV disease has been associated with increased thickness and volume of EAT [12]. EAT from these patients shares some alterations with those ascribed to CAD patients [3,4]. In this context, it is important to distinguish whether the features are due to diabetes itself or to the co-existence of CAD.

EAT stores energy in form of nonesterified fatty acids (NEFA) to provide primary fuel for the cardiac muscle, a process that must be tightly controlled, avoiding overwhelming/excessive levels of NEFA in coronary circulation. Compared to subcutaneous adipose tissue, EAT is a richer source of NEFA and sphingolipids, mainly in CAD patients [13], in whom there is increased content of ceramides (Cer), diacylglycerides (DAG), and monoacylglycerides and decreased unsaturated NEFA. The EAT of patients with type 2 diabetes mellitus has also shown an increase in specific saturated NEFA and a decrease in unsaturated NEFA [14]. When the storage capacity of adipocytes is exceeded, these potentially inflammatory lipids may be released, which can affect the myocardial function in CAD and diabetic patients.

Taken together, the lipid species and proteins released by EAT in diabetic patients can exert a harmful action on the myocardium, which shares microcirculation with EAT, because there is a direct interaction, with no fascia separating both tissues. It has been reported that the secretome of EAT from diabetic patients dampers cardiomyocyte contractibility and mitochondrial β-oxidation, which are both effects mediated by lipids released by EAT [15]. Among those lipid species inducing alterations in cardiomyocytes, some studies have suggested the involvement of saturated NEFA and Cer. Specifically, palmitic acid induces reactive oxygen species formation, endoplasmic reticulum stress, and apoptotic pathways in the cardiomyocyte line AC16 [16,17]. These actions may be partly mediated through Cer biosynthesis, since saturated NEFA, and particularly palmitate, are precursors of this lipid mediator. Cer induces insulin resistance and mitochondrial dysfunction in the H9c2 cardiomyocyte line [18] as well as inflammation [19,20] and lipotoxicity [21] in AC16 cells. Recently, the role of ceramide in oxidative stress and the inflammatory state has been extensively reviewed [22–24].

High-density lipoprotein (HDL) and apolipoprotein J (apoJ) are protective molecules of the myocardium [25]. ApoJ, also known as clusterin, protects cardiac cells against damage derived from infarction or myocarditis by regulating several intracellular pathways related to oxidation, inflammation, glucose metabolism, and apoptosis. In particular, apoJ has been reported to protect cardiomyocytes from cell death and inflammation [25–29]. Diabetic patients usually show low plasma levels of HDL with diminished apoJ content [30] and impaired anti-atherogenic properties [31]. However, the effect of both molecules in counteracting the putative deleterious effects of EAT on cardiomyocytes has not previously been reported.

The metabolic crosstalk between EAT and the myocardium, and the effect of this interaction, has not been fully elucidated, and in the case of diabetic patients, limited information is available. In this scenario, we conduct a proof-of-concept study to ascertain the morphological characteristics, release of molecules, and deleterious effects of EAT secretomes from type 2 diabetic patients, discriminating between patients with or without CAD on cardiomyocyte function. In addition, we assess the putative protective role of apoJ and HDL against the deleterious effects of EAT secretomes.

2. Methods

2.1. Patients

EAT explants were obtained under sterile conditions during planned cardiac surgical interventions (heart valve surgery or coronary bypass) by the cardiac surgery team of the Hospital de la Santa Creu i Sant Pau. Patients who have suffered an acute coronary syndrome in the last three months and those having infectious or inflammatory diseases were excluded from the study. Patients were classified into nondiabetic (ND) and diabetic (DM) groups. DM patients were defined as those with a fasting glucose level ≥126 mg/dl, current use of DM medication, or reported diagnosis of DM. DM patients were divided into groups according to the presence or absence of previous CAD, with these groups named DM-C and DM, respectively. All subjects provided written informed consent before participating in the study, and the protocol was approved by the ethical committee of the Hospital de la Santa Creu i Sant Pau (IIBSP-REL-2017–27). The study was performed in accordance with the Helsinki Declaration. The anthropometric and clinical characteristics of the patients included in the study are shown in Supplemental Table 1.

2.2. EAT explants analysis

EAT was cut into pieces of 100 mg. One piece of EAT was frozen for gene expression studies, and another was paraffined for immunohistochemistry (IHC) analysis. Macrophage infiltration was assessed by CD68 immunostaining (anti-CD68 clone PG-M1 antibody, Dako chemicals) in deparaffined samples. Adipocyte size and the number of infiltrated macrophages were evaluated by microscopy using AxioVision V 4.8.1.0 image analysis software (Zeiss, Jena, Germany). The remaining EAT pieces were incubated in a 12-well cell plate at 37ºC and 5% CO_2 in DMEM medium supplement with 1% penicillin-streptomycin (Biowest, Nuaille, France) for 24 h. Cell supernatant (secretome) was collected and kept at −80ºC until analysis. LDH activity was measured in the supernatant according to the manufacturer's instructions (LDH kit, Roche, Merck Life Sciences)

2.3. Lipidomic analysis

Levels of sphingolipids in secretome were evaluated by liquid chromatography–mass spectrometry (LC-SM) in the CIBERDEM-Metabolomics Platform of Universitat Rovira i Virgili. Briefly, lipid extraction from 200 µl of secretome (100 mg of EAT cultured in 1 mL of medium) was performed, as previously described [32]. Lipids were reconstituted in 150 µL of MeOH:toluene (9:1) and transferred to LC-MS vials. Lipid extracts (5 µL) were injected in a UHPLC system (1290 Agilent) coupled with a triple quadrupole (QqQ) mass spectrometer (6490 Agilent Technologies) operated in positive electrospray ionization mode. The instrument was set to acquire in MRM mode. Lipids were separated using C18-RP (ACQUITY UPLC BEH 2.1 × 150 mm, 1.7 µm, Waters) chromatography at 65ºC and at a flow rate of 0.4 mL/min. The solvent system consisted of acetonitrile:water (60:40; A) in 10 mM ammonium formate and isopropanol:acetonitrile (90:10; B) in 10 mM ammonium formate. The gradient elution started at 15% B and went to 30% from 0 to 2 min, 48% B from 2 to 2.5 min, 82% B from 2.5 to 11 min, and 99% B from 11 to 11.5 min. Cer 14:0, Cer 16:0, Cer 18:0, Cer 20:0, Cer 22:0, Cer 24:0, and Sph 18:1 relative concentrations were quantified by this method. Total Cer was considered to be the sum of all the Cer species evaluated.

For NEFA analysis, volatile fatty acids methyl ester derivatives (FAMEs) were first obtained as described in [33,34]. Briefly, 20 µl of the sample was mixed with internal standard solution and methanol and incubated at 4ºC for 10 min to extract the fatty acids and help the protein precipitation. After this, a derivatization step was conducted using boron trifluoride 14% in methanol. Afterwards, the obtained FAMEs

were extracted by liquid–liquid extraction using hexane before being injected into the GC-MS system. Chromatographic analysis was based on the determination of the 37 FAMEs included in the Food Industry FAME Mix. FAMEs were separated on a HP-88 column (100 m × 250 μm × 0.25 μm) using a temperature program between 140ºC and 240ºC at 1 mL/min and using He as a carrier gas. Ionization was carried out by electronic impart (70 eV) and a mass analyzer operating in selected ion monitoring mode.

2.4. Incubation of secretome with cardiomyocytes

AC16 human cardiomyocyte line (Sigma-Aldrich, Sant Louis, Missouri, USA) was used to study the effect of secretome on cardiomyocytes in the presence or absence of HDL, apoJ, Cer, palmitic acid (PA), or myriocin. HDL (1063–1210 g/mL) was isolated from the pooled plasma of healthy volunteers by sequential flotation ultracentrifugation [35]. ApoJ was expressed in human embryonic kidney 293 T cells (HEK293T) transfected with the pcDNA4.0™ vector containing human APOJ cDNA and isolated by Ni-affinity chromatography with fast protein liquid chromatography, as described in [36].

AC16 cells were grown according to the manufacturer's recommendations. The growth medium for AC16 was DMEM-F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin from Biowest. Prior to the incubation with stimuli, AC16 cells were seeded (40,000 cells/well) for 24 h in 12-well plates with DMEM-F12 medium and supplemented with 1% FBS and 1% penicillin-streptomycin (deficient medium). Then, AC16 cells were incubated with different stimuli. Secretome diluted at 1/20 in the presence or absence of HDL at 60 mg apoA-I/L or apoJ at 50 mg/L was added to the cells for 24 h. In another experiment, AC16 cells were incubated with Cer 16:0, Cer 24:1 (5 μM and 20 μM), and PA (100 μM and 300 μM) in the presence or absence of myriocin (1 μM) for 24 h in deficient medium supplemented with 2% bovine serum albumin. The Cer 16:0, PA, and myriocin were from Cayman Chemicals (Ann Arbor, Minnesota, USA) and the Cer 24:1 was from Avanti Polar Lipids (Alabaster, Alabama, EUA). After incubation, the cell supernatants were collected and stored at −80 ºC until analysis, and the cells were treated as explained in the corresponding sections.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The release of interleukin 6 (IL6) and monocyte chemoattractant protein 1 (MCP1) was quantified in the AC16 supernatants by ELISA kits (Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer's instructions. ApoJ released by EAT explants in the secretome was measured using an ELISA kit (Mabtech, Stockholm, Sweden) according to the manufacturer's instructions.

2.6. Apoptosis assay

The cell apoptosis assay was evaluated in the AC16 cells after incubation with stimuli with an eBiosciences Annexin V-FITC Apoptosis detection kit (Invitrogen), as previously described [32].

2.7. Real-time polymerase chain reaction (RT-PCR)

RNA was extracted from 100 mg of frozen EAT or from 1×10^6 cells cultured in six-well plates after incubation with the secretome diluted at 1/20 for 4 h. RNA extraction from EAT was performed using the RNeasy Lipid Tissue Mini (Qiagen, Hilden, Germany). RNA extraction from the AC16 cells was done using the EZ-10 DNAaway RNA miniprep kit (BioBasic; Markham, Canada). Reverse transcription was performed with 0.5 μg of RNA using EasyScript First-Stand cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China). Quantitative RNA analysis was conducted using RT-PCR in a CFX96 system (BioRad, Hercules, CA, USA). All human gene expression assays were from Applied Biosystems (Beverly, MA, USA). The genes quantified in EAT were as follows: *MCP1* (Hs00234140_m1), *IL6* (Hs00174131_m1), *IL1β* (Hs01555410_m1), *IL10* (Hs00961622_m1), *IL8* (Hs00174103_m1), glucose transporter type 4 (*GLUT4*; Hs00234140_m1), retinol-binding protein 4 (*RBP4*; Hs00924047_m1), perilipin 2 (*PLIN2*; Hs00605340_m1), cluster of differentiation 36 (*CD36*; Hs00354519_m1), peroxisome proliferator-activated receptor gamma (*PPARG*; Hs01115513_m1), apoJ (*CLU*; Hs00156548_m1), fatty acid-binding protein 4 (*FABP4*; Hs01086177_m1), carnitine palmitoyltransferase I (*CPT1A*; Hs00912671_m1), neutral sphingomyelinase activation associated factor (*NSMASE*; Hs01060813_m1), serine palmitoyltransferase 2 (*SPT2*; Hs01027014_m1), ceramide synthase 2 (*CERS2*; Hs00604577_m1), ceramide synthase 5 (*CERS5*; Hs00332291_m1), and toll-like receptor 4 (*TLR4*; Hs00152939_m1G). *MCP1* and *IL6* expression was also measured in the AC16 cells. Human actin β (*ACTB*) was used as the internal control (Hs99999903_m1) in both the EAT and the AC16 cell analyses.

2.8. Statistical analysis

Prism 8.0 software (GraphPad, San Diego, California, USA) was used for the statistical analyses. Kruskal-Wallis test with Dunn's multiple comparisons post-test was used to compare more than two groups. To confirm these differences, data were analyzed using the non-parametric tests Mann-Whitney U test (for unpaired samples) or the Wilcoxon test (for paired samples). Morphological characteristics, gene expression analysis, ELISA and cytotoxicity tests were performed in triplicate. Results were expressed as mean ± SD. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. EAT morphological features are altered in diabetic patients

EAT from diabetic patients, particularly from the DM-C group, showed larger adipocyte size and increased macrophage infiltration, evaluated as CD68 staining, compared with the ND group (Supplemental Figure 1, Supplemental Table 2). Although the number of infiltrated macrophages was similar in the DM and DM-C samples, in the latter, crown-like structures composed of macrophages surrounding dead or dying adipocytes were more abundant than in the DM samples [37]. Since the high size of adipocytes and inflammation can lead to cell mortality and crown-like structures are also associated with cell death, EAT explants were cultured, and after 24 h, the released LDH activity was evaluated in the secretome as an indirect measurement of cell mortality. EAT from the DM-C samples showed the highest LDH activity in the secretome (Supplemental Table 2).

3.2. EAT from diabetic patients shows an altered gene expression

The abovementioned alterations in EAT from the DM and DM-C samples could reflect changes in gene expression. According to the increased macrophage infiltration observed in the diabetic samples, enhanced expression of several inflammation-related genes was observed in the EAT of the diabetic subjects. This situation was particularly exacerbated in the presence of coronary disease (Fig. 1A–E). In addition, some statistically significant alterations in genes related to glucose and lipid metabolism were achieved. EAT from the diabetic patients showed decreased expression of *CD36*, *GLUT4*, and *RBP4* and increased expression of *PLIN2* (Fig. 1F–I). No significant differences were found in these genes between the DM and DM-C samples.

Supplemental Figure 2 shows the expression of other genes related to lipid metabolism that did not show statistical differences between groups, including *PPARG*, *FABP4*, *CPT1A*, *NSMASE*, *SPT2*, *CERS2*, *CERS5*, and *TLR4*.

Another protein whose expression we were interested in was apoJ. Its expression was increased in the diabetic samples, albeit only in the

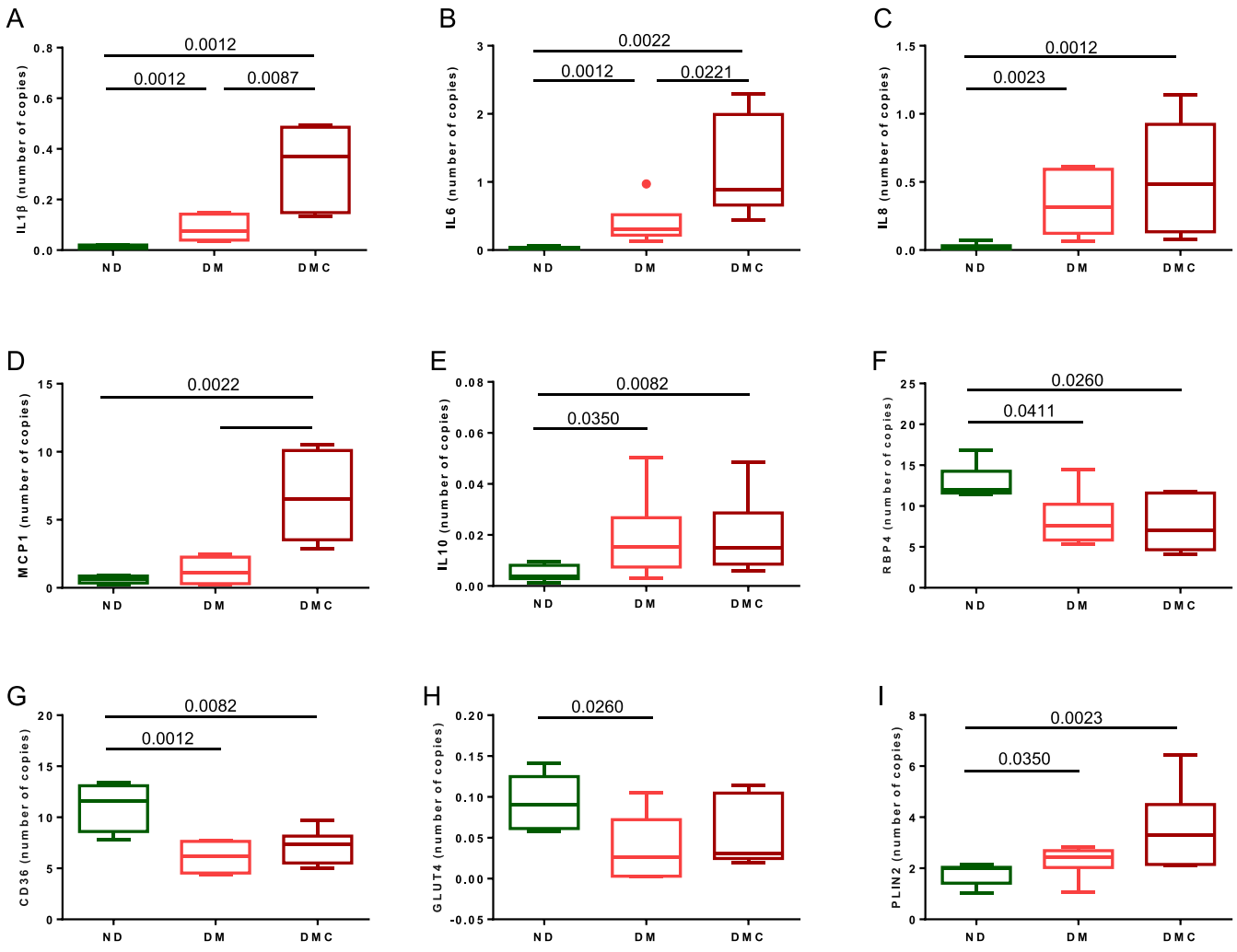

Fig. 1. Expression of genes related to metabolism and inflammation in EAT. RNA was extracted from 100 mg of EAT from nondiabetic patients (ND, $n = 7$), diabetic patients (DM, $n = 6$), and patients with diabetes plus coronary heart disease (DM-C, $n = 6$). RT-PCR was performed as described in the Methods and expression was relativized to β-actin expression. Data are expressed as mean ± SD. Horizontal lines indicate statistically significant differences.

absence of coronary disease, which was unexpected (Fig. 2A). This behavior was confirmed by measuring the concentration of apoJ released in the secretome (Fig. 2B).

As a whole, these results suggest that the existence of diabetes promotes relevant alterations in the expression of genes related to inflammation and metabolism, leading to the release of molecules with a harmful effect on surrounding cells.

3.3. EAT from diabetic patients releases specific NEFA and Cer species

The high adipocyte size and increased expression of *PLIN2*, a protein involved in the formation of intracellular lipid droplets, indicated that the adipocytes from the diabetic patients accumulated lipids. Part of these lipids could be hydrolyzed and fatty acids or other lipid species released into the extracellular milieu. We assessed NEFA and ceramide

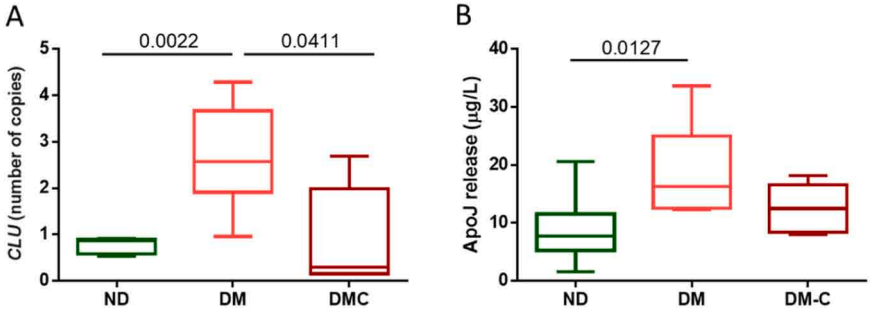

Fig. 2. Gene expression (A) and release (B) of apoJ from EAT. RNA was extracted from 100 mg of EAT from nondiabetic patients (ND, $n = 7$), diabetic patients (DM, $n = 6$), and patients with diabetes plus coronary heart disease (DM-C, $n = 6$). ApoJ concentration in the culture medium was determined by ELISA ($n = 8$ for each group). RT-PCR (A) and ELISA (B) were performed as described in the Methods. Data are expressed as mean ± SD. Horizontal lines indicate statistically significant differences.

species in the secretome because of their well-described involvement in promoting cardiomyocyte dysfunction [16–19,21]. The secretomes from the DM-C group had higher NEFA content compared to the ND and DM groups (Fig. 3A), but those from the DM group without coronary disease did not release more NEFA compared to the ND group. This increased content in the EAT from the DM-C samples was observed in saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) (Fig. 3B). Fig. 3C shows the individual NEFA species detected in the secretomes. Specifically, myristate (C14:0), palmitate (C16:0), stearate (C18:0), oleate (C18:1), eicosenoate (C20:1), and arachidonate acids (C20:4) were statistically increased in the secretome from the DM-C group, compared to the ND and DM groups.

Regarding sphingolipid-derived molecules, no differences in sphingosine and total Cer content were found among the groups (Fig. 4A), nor were differences found when the ceramide species were considered individually (Supplemental Figure 3). However, when analyzing the ratios of the different ceramide species, which have been associated with cardiometabolic risk [38–40], significant differences were observed (Figs. 4B and 4C). In this context, ceramide 24:0 (Cer24:0) is considered a protective molecule, while Cer16:0, Cer18:0, and Cer24:1 exert pro-apoptotic and inflammatory properties [41]. Specifically, the Cer16:0/Cer24:0 and Cer24:1/Cer24:0 ratios were increased in the secretome of the DM samples, compared to the ND samples. Surprisingly, these ratios were not increased in the DM-C samples, compared to the DM ones.

3.4. EAT secretome in diabetic patients induces inflammatory cytokine release and cytotoxicity in cardiomyocytes

Altogether, the findings observed from the EAT of diabetic patients and their secretome suggest the generation of an inflammatory/cytotoxic surrounding environment that may contribute to cardiomyocyte dysfunction. Fig. 5 shows IL6 and MCP1 expression and release in cardiomyocytes after incubation with secretomes. Cardiomyocytes showed a higher expression of these genes related to inflammation (Figs. 5A and 5B) when incubated with the secretome of EAT from diabetic patients but not when incubated with EAT from ND ones. The increased cardiomyocyte expression of *IL6* and *MCP1* genes induced by the secretome of DM samples was accompanied by the increment of these molecules in the cell supernatant (Figs. 5C and 5D). Surprisingly, the secretomes from

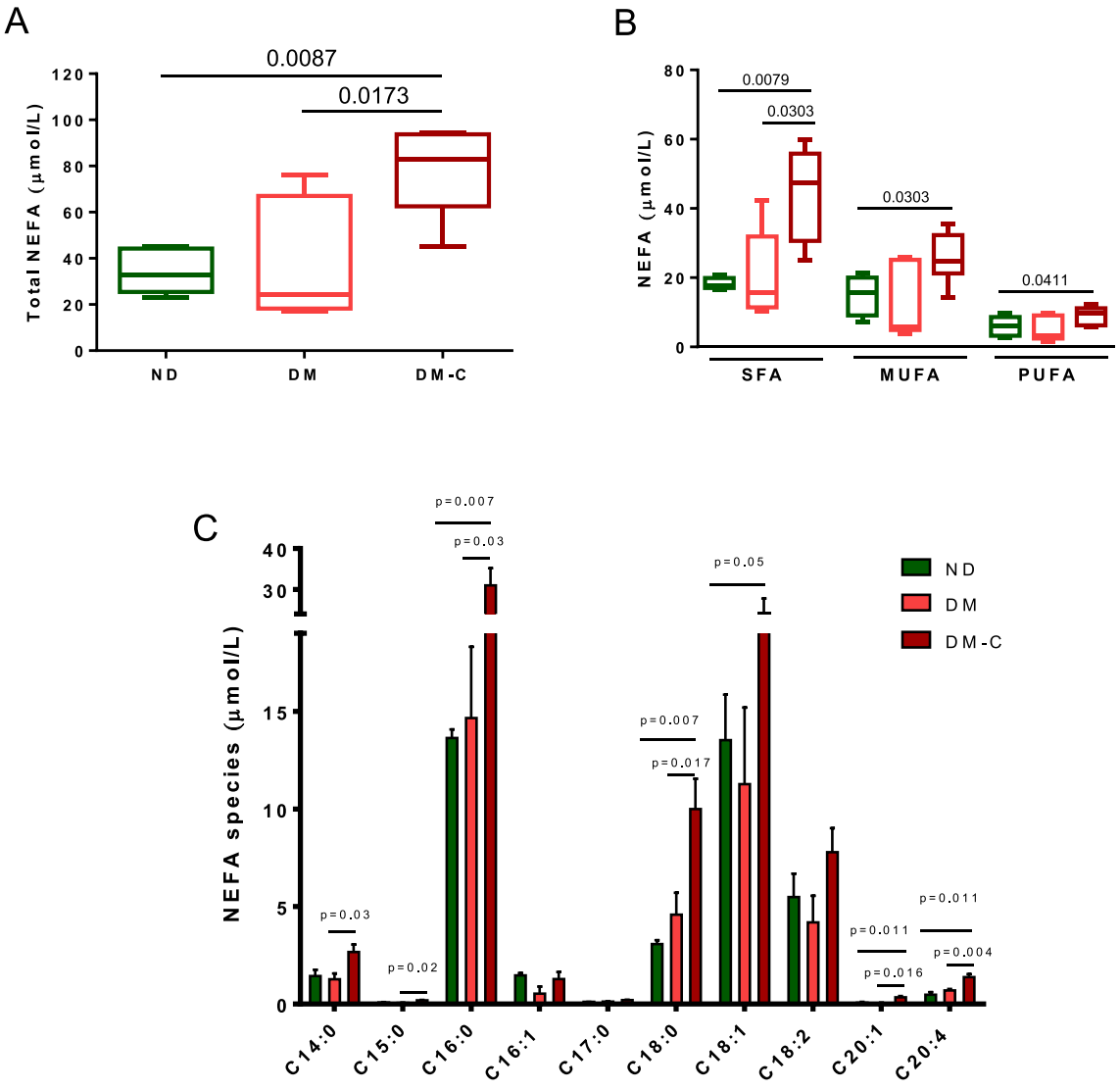

Fig. 3. NEFA content (A: total NEFA; B: NEFA according to saturation level; C: individual NEFA species) of secretomes from EAT. EAT explants (100 mg) were cultured for 24 h and the supernatant collected. Lipid species in the secretome were evaluated by mass spectrometry, as described in the Methods. Data are expressed as mean ± SD ($n = 6$). Horizontal lines indicate statistically significant differences.

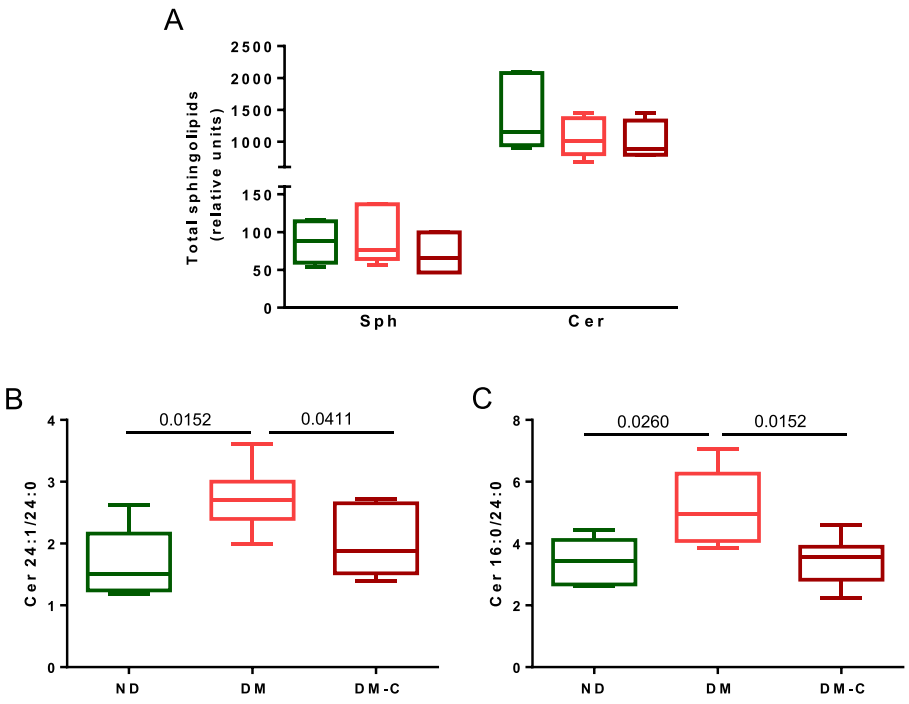

Fig. 4. Ceramide and sphingosine content (A) and ceramide species ratios (B and C) in secretomes from EAT. EAT explants (100 mg) were cultured for 24 h and the supernatant collected as described in the Methods. Sphingosine and ceramide species in the secretome were evaluated by mass spectrometry, as described in the Methods. Data are expressed as mean ± SD ($n = 6$). Horizontal lines indicate statistically significant differences.

the DM samples promoted the greatest inflammatory effect, whereas those from the DM-C samples induced a release of both cytokines halfway between that of the ND and DM samples.

Regarding cytotoxic effects, secretomes from the ND group did not induce apoptosis or necrosis in AC16. In contrast, those from the DM group, and particularly from the DM-C group, promoted higher necrotic effect than those from the ND group, without a significant effect on early or late apoptosis (Fig. 5 E).

3.5. Cer and PA induce inflammatory and necrotic effects on cardiomyocytes

The inflammatory and cytotoxic effects induced by the secretomes from diabetic patients on cardiomyocytes and the presence of bioactive lipids in such secretomes suggest a role for PA, Cer16:0, and Cer24:1. Therefore, we evaluated the putative role of these individual lipid species in promoting inflammation and apoptosis/necrosis in AC16 cells. Indeed, PA, Cer16:0, and Cer24:1 induced IL6 and MCP1 release in cardiomyocytes (Fig. 6). PA-induced cytokine release was significantly inhibited by myriocin, an inhibitor of serine palmitoyl transferase.

Regarding apoptosis, PA had no effect on early apoptosis but induced late apoptosis and necrosis in AC16 cells, with both effects partly counteracted by myriocin (Fig. 7). In contrast, Cer species did not induce a significant effect on necrosis but promoted early apoptosis, particularly Cer16:0. Cer16:0 also induced late apoptosis, but Cer24:1 had no effect. No ceramide had any effect on necrosis (Fig. 7).

3.6. HDL and apoJ revert the inflammatory and apoptotic effects of the EAT secretome

The addition of apoJ lowered the release of MCP1 by approximately 40%–70% and that of IL6 induced by EAT secretomes by 20%–60% (Fig. 8A and B). HDL was also effective in lowering the release of MCP1 (30%–60%) but had no significant effect on IL6 release (Fig. 8A and B). No statistical differences between groups were observed. In addition, apoJ and HDL significantly inhibited the necrotic effect of secretomes from the DM and DM-C patients but had no effect on the ND samples, since they had not promoted any apoptotic effect (Fig. 8C). Neither HDL nor apoJ alone induced changes in inflammation or apoptosis/cytotoxicity (data not shown). Taken together, apoJ and HDL exerted a protective role by counteracting the harmful inflammatory and cytotoxic effects of secretomes from diabetic patients on cardiomyocytes.

3.7. ApoJ is expressed and released by cardiomyocytes in response to the EAT secretome from diabetic patients

The expression and release of apoJ was also assessed in AC16 cardiomyocytes incubated with secretomes (Fig. 9). All secretomes stimulated the expression and release of apoJ, compared to blank cells. The secretomes of the DM-C samples significantly increased the expression of apoJ, compared to that of the ND and DM samples. In contrast, the release of apoJ induced by DM-C secretomes was lower than that induced in the DM samples, perhaps reflecting dysfunction in the secretion of apoJ from cardiomyocytes when stimulated with DM-C secretomes.

4. Discussion

EAT is the visceral fat depot over the heart that protects and supplies fatty acids to the myocardium. However, the increment of EAT volume in diabetic patients has been associated with the development of CV disease [12]. This action could be partly due to the active release of lipid mediators with deleterious effects on the surrounding cells of coronary arteries and the myocardium. The current study provides novel information regarding the qualitative characteristics of EAT in diabetic patients and the effect of its secretome on human cardiomyocytes. Secretomes from these patients induced inflammation and cytotoxicity in AC16 cardiomyocytes, and both effects were partly mediated by the release of NEFA and Cer species. Our data showed that apoJ, which is also expressed and released by EAT, may contribute to minimize/reduce

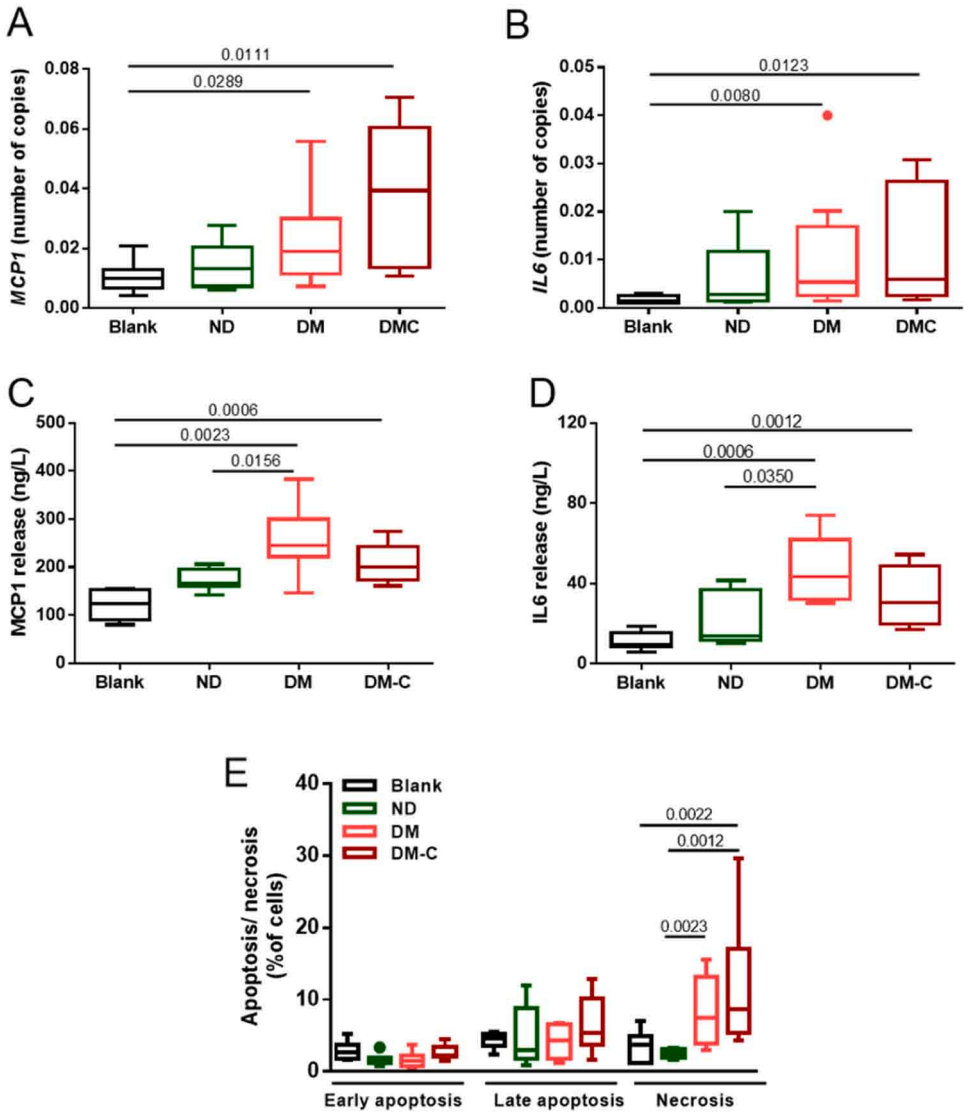

Fig. 5. MCP1 (A and C) and IL6 (B and D) expression and release and cytotoxicity (apoptosis + necrosis) (E) induced by EAT secretomes in AC16 cells. For expression analysis, AC16 cells were incubated with the secretome (diluted 1/20) of the EAT from nondiabetic patients (ND), diabetic patients (DM), and patients with diabetes plus coronary heart disease (DM-C) ($n = 7$ for each group) for 4 h. Afterwards, RNA was extracted from cells and RT-PCR performed from the cDNA, as described in the Methods. The expression of all the probes were relativized to β-actin expression and expressed as number fold vs. blank (basal expression of AC16 cells not incubated with secretome) (A and B). For cytokine release and apoptosis analysis, cells were incubated with the secretome of EAT from the ND ($n = 7$), DM ($n = 7$), and DM-C ($n = 7$) patients for 24 h. Afterwards, MCP1 and IL6 release was evaluated in the supernatant by ELISA (C and D) and apoptotic/necrotic cells were quantified by annexin V and/or propidium iodide staining by flow-cytometry (E), as described in the Methods. Data are expressed as mean ± SD. Horizontal lines indicate statistically significant differences.

these deleterious actions.

The relationship between EAT and CV disease is not only limited to EAT volume but also to the qualitative characteristics of this tissue. In this regard, some studies have reported EAT from patients with CAD to show morphological alterations [3,42], an inflammatory profile, and changes in gene expression [5–9]. However, information on qualitative alterations in the EAT of diabetic patients and its relationship with CV risk is scarce. In this context, our study showed some morphological differences in the EAT of diabetic patients that can vary depending on the presence of coronary disease. Thus, adipocytes in the DM samples displayed a size similar to those from the nondiabetic samples, but they presented increased macrophage infiltration. In contrast, EAT samples from the DM-C patients had both higher sized adipocytes and increased macrophage infiltration. This was reflected in the high expression of inflammatory molecules. Such increased expression was especially enhanced in the coronary samples, which concurs with previous studies [3,4], contributing then to an inflammatory state of EAT. Since the number of infiltrated macrophages was similar in the DM and DM-C samples, the higher expression of cytokines in the latter could indicate the increased inflammatory potential of these macrophages. In agreement, EAT samples from the DM-C patients showed more crown-like structures, which are histologic hallmarks of the pro-inflammatory process in adipose tissue [37]. These structures are composed of macrophages surrounding dead or dying adipocytes [43], which also concurs with the increased mortality observed in the DM-C samples compared with the DM ones. This observation agrees with previous observations of EAT from coronary patients [44].

On the other hand, EAT from the DM patients showed an altered expression of genes related to insulin resistance, fatty acid uptake, and lipid accumulation. PLIN2 is a key protein for the formation of intracellular lipid droplets for the storage of lipids. Therefore, the increased *PLIN2* expression in EAT from the DM patients, and especially from the

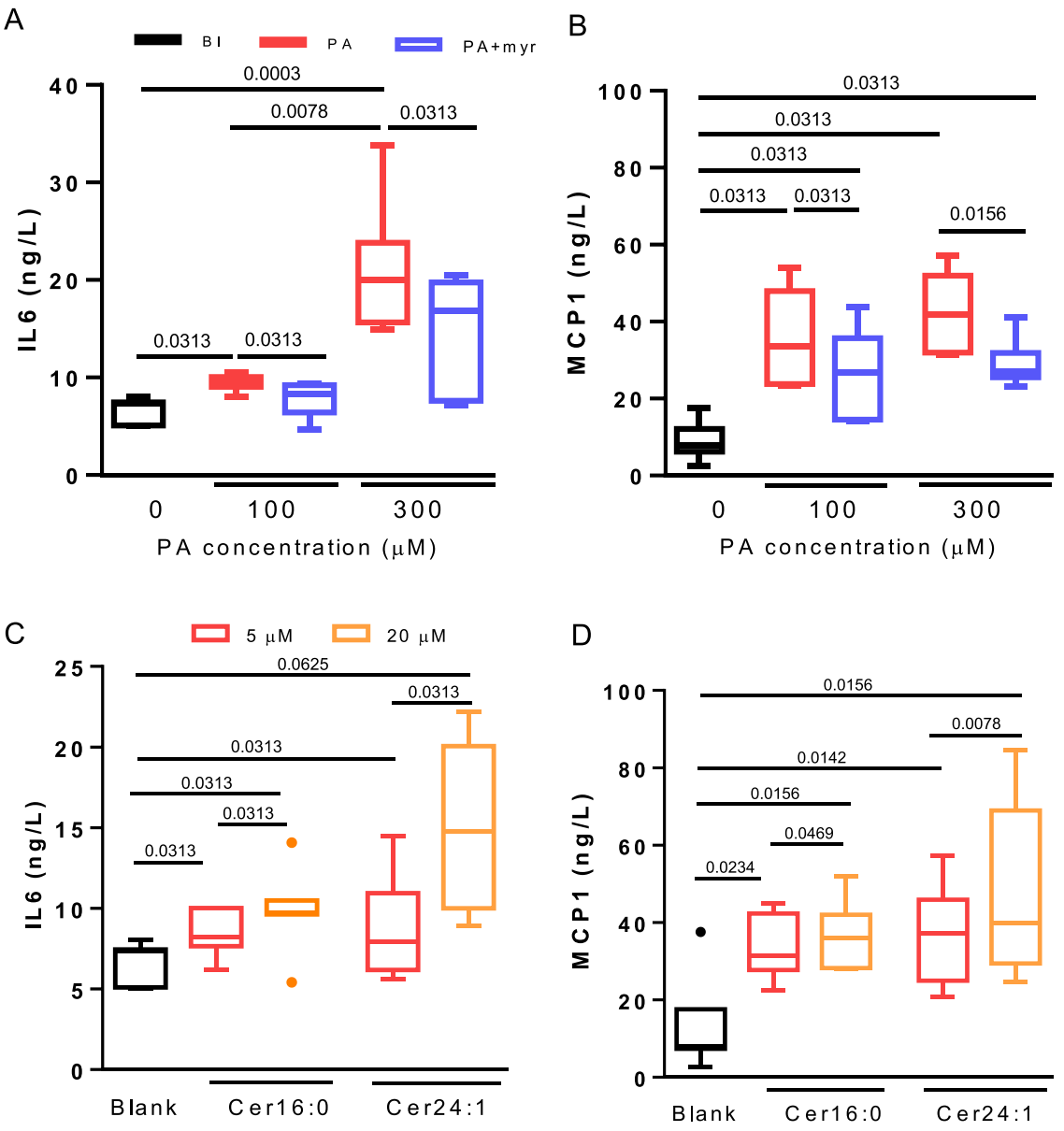

Fig. 6. Inflammatory effect of PA (A and B) and Cer species (C and D) on AC16 cardiomyocytes. Before their addition to cells, PA, Cer 16:0, and Cer 24:1 were incubated with albumin for 2 h. AC16 cells were then incubated with the indicated concentrations of lipids for 24 h. IL6 (A and C) and MCP1 (B and D) were measured in the supernatant by ELISA, as described in the Methods. $n = 7$ for each group. Data are expressed as mean ± SD. Horizontal lines indicate statistically significant differences.

DM-C patients, probably contributed to higher TG accumulation and the ensuing larger size of the adipocytes found in these samples. In contrast, the decreased expression of the main transporter of glucose, *GLUT4*, in the DM samples may have been related to insulin resistance in the EAT of these subjects, as described in [45–48]. In addition, the expression of the fatty acid receptor *CD36* was down-regulated in the DM samples, suggesting a disturbance of fatty acid homeostasis and insulin resistance in these patients. In accordance, it has been suggested that *CD36* expression in adipose tissue develops a protective role against insulin resistance in liver and muscle [49].

Besides alterations in gene expression, our data also showed that the secretomes from the DM samples presented differences in the release of NEFA and Cer species, compared to the ND samples. Interestingly, such differences were not the same in DM and DM-C samples. Previous studies reported the EAT from CAD and type 2 diabetic patients to show increased content of lipid mediators, including Cer and saturated NEFA [13,14,50]. Under the situation of insulin resistance, EAT has been described to release NEFA [51], such as PA, which promotes lipid accumulation and other alterations in cardiomyocyte functionality, leading to diabetic cardiomyopathy [50]. In our study, EAT from the DM-C samples, but not from the DM ones, released more total NEFA, particularly SFA, with PA being the most abundant and also increased species, compared to the ND samples. In contrast, EAT from the DM samples released NEFA at a similar level to that from the ND samples, but it released altered ratios of Cer 16:0/24:0 and Cer 24:1/24:0. Indeed, the plasma levels of Cer 16:0 and Cer 24:1 are associated with adiposity and diabetes [52,53]. In contrast, C24:0 shows a negative relationship with CV risk [40,54]. Thus, high Cer 16:0/24:0 and Cer 24:1/24:0 ratios have been associated with CV risk and worse CV outcomes [54–57]. Since Cer is a well-known inflammatory and lipotoxic molecule [21,58,59], the release of these Cer species by the secretomes from the DM samples may be feasibly involved in the induction of inflammation and

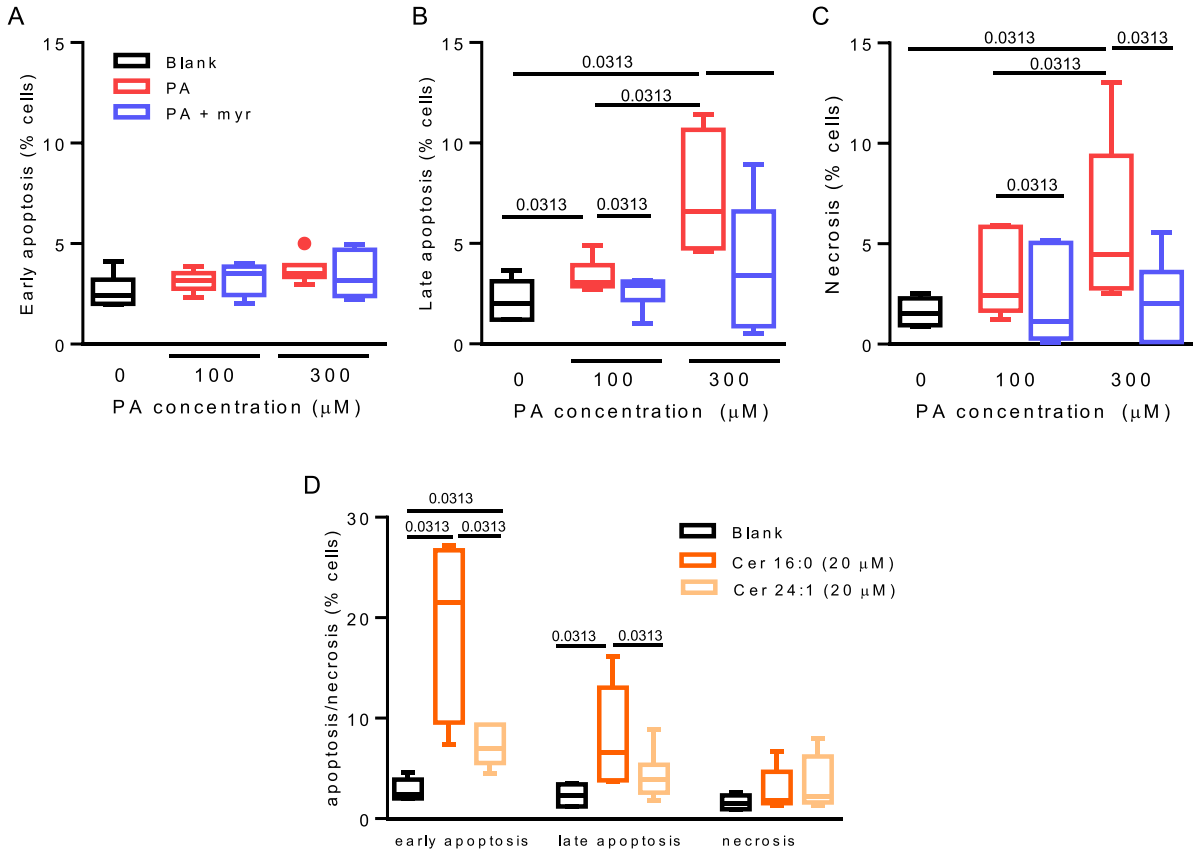

Fig. 7. Cytotoxic effect of PA (A–C) and Cer species (D) on AC16 cells. Before their addition to cells, PA, Cer 16:0, and Cer 24:1 were incubated with albumin for 2 h. AC16 cells were then incubated with the indicated concentrations of the lipids for 24 h. Apoptosis and necrosis were evaluated by staining with annexin-FITC and propidium iodide, respectively, and flow cytometry detection. $n = 7$ for each group. Data are expressed as mean ± SD. Horizontal lines indicate statistically significant differences.

cytotoxicity in AC16 cardiomyocytes.

Therefore, these alterations observed in EAT samples from the diabetic patients, both in gene expression and lipids, may cause harmful consequences for neighboring cardiomyocytes. We checked this possibility by incubating the secretomes from EAT with AC16 cardiomyocytes, a cell model that reproduces the functional characteristics of physiological cardiomyocytes [60]. This cell line has been described to actively release inflammatory molecules, such as MCP1 and IL6, in response to different stimuli [61,62]. In accordance, the secretomes from the DM and DM-C samples, but not those from the ND ones, induced the release of MCP1 and IL6 from AC16 cardiomyocytes. In addition, AC16 cells, when incubated with secretomes from diabetic patients, and mainly those of DM-C, became necrotic.

These results suggest that the high NEFA content in secretomes from the DM-C samples may mediate the necrotic effect induced on AC16 cardiomyocytes. However, this effect could be directly mediated by SFA, such as palmitate, or indirectly after the conversion of palmitate to ceramide by serine palmitoyl transferase 1. Moreover, the altered ratio of ceramides in the secretomes from the DM samples could be involved in the inflammatory and cytotoxic effect on cardiomyocytes. To test this hypothesis, *in vitro* experiments adding purified PA, Cer16:0, and Cer24:1 to AC16 cells were performed. PA induced late apoptosis and necrosis but had no effect on early apoptosis, a pattern similar to that observed after the addition of secretomes from the DM-C samples. This cytotoxic effect agrees with the previously described effect of PA and other SFA in cardiomyocytes [16,17,63,64]. In contrast, Cer16:0 and Cer24:1 strongly stimulated early apoptosis, but only Cer16:0 induced late apoptosis, and neither Cer16:0 nor Cer24:1 had an effect on necrosis. These findings suggest a minor role for these ceramides in the cytotoxic effect of secretomes from diabetic samples, which is expressed mainly as necrosis. In turn, PA seems to play a major role in the cytotoxicity induced by EAT secretomes from diabetic patients with coronary disease [51–54].

Regarding inflammation, purified PA also induced IL6 and MCP1 release from AC16 cardiomyocytes. This finding seemingly disagrees with the results observed after the incubation of secretomes with cardiomyocytes, since these cytokines were not differentially induced by the DM-C samples, compared to the ND ones. This discrepancy could be a consequence of the strong induction of necrosis by DM-C secretomes, which render less “alive/active” cells able to secrete cytokines and apoJ.

Both the apoptotic and inflammatory effects induced by PA were partially inhibited by the addition of myr, an inhibitor of Cer biosynthesis, suggesting a role for Cer in mediating these effects, as previously proposed [65–67]. The inflammatory effect was reproduced by the exogenous addition of proatherogenic Cer16:0 and Cer24:1 to cultured AC16 cells. Both ceramide species induced inflammation, but Cer24:1 had a stronger effect than Cer16:0 on cytokine release. These findings concur with previous studies pointing to ceramides as inflammatory mediators [58,59].

One of the proteins whose expression was increased in EAT from the DM samples was apoJ, whose concentration was also increased in secretomes. This apolipoprotein is partially transported in plasma bound to HDL and other lipoproteins, but its predominant form in tissues is as free form. ApoJ acts as an extracellular chaperone that prevents protein misfolding and preserves protein function and is released by different tissues in response to injury [68,69]. Specifically in the heart, apoJ has been reported to protect cardiomyocytes from cell death and inflammation induced by oxidative stress via the Akt/GSK-3β signaling

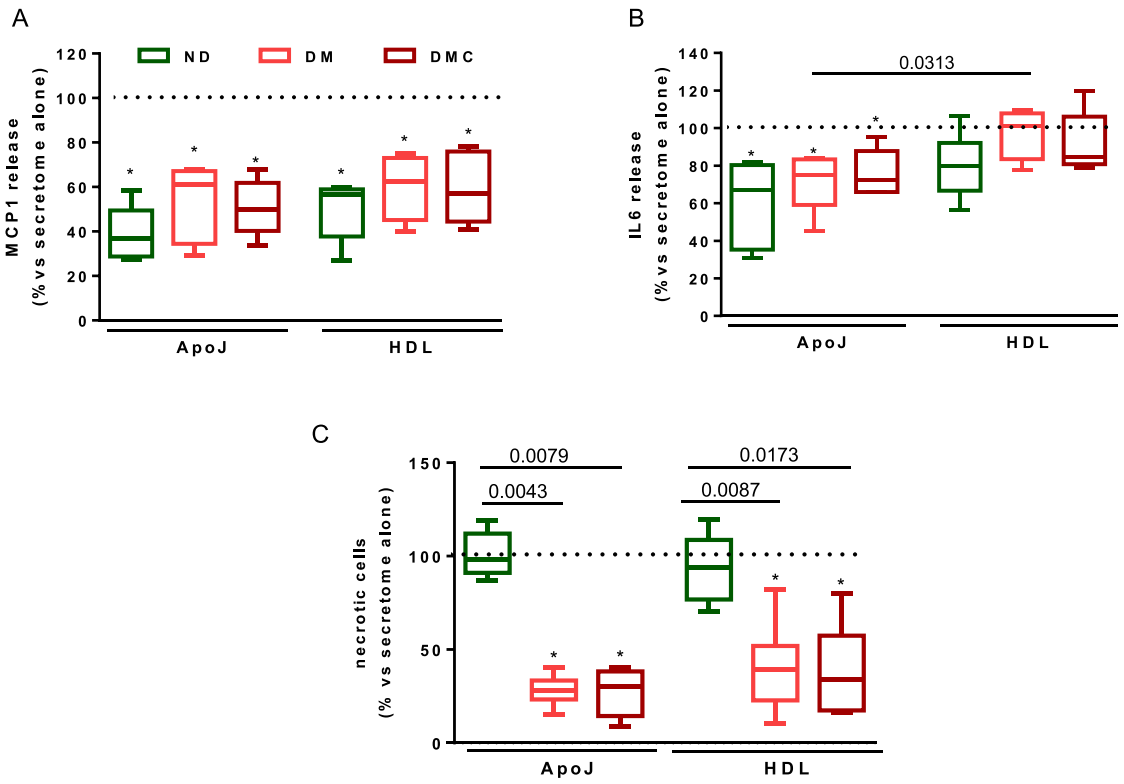

Fig. 8. Effect of apoJ and HDL in preventing secretome-induced MCP1 and IL6 release and cytotoxicity in AC16 cells. Cells were incubated with the secretome of EAT from nondiabetic patients (ND, $n = 7$), diabetic patients (DM, $n = 7$), and patients with diabetes plus coronary heart disease (DM-C, $n = 7$) for 24 h and apoJ or HDL, as described in the Methods. Afterwards, MCP1 and IL6 release was evaluated in the supernatants by ELISA (A and B) and apoptotic/necrotic cells were quantified by annexin V and/or propidium iodide staining by flow-cytometry (C), as described in the Methods. Data are expressed as percentage vs. cells incubated only with secretome (indicated by the dotted line) (mean ± SD). Horizontal lines indicate statistically significant differences between groups. * indicates statistically significant differences vs. cells incubated with secretome alone.

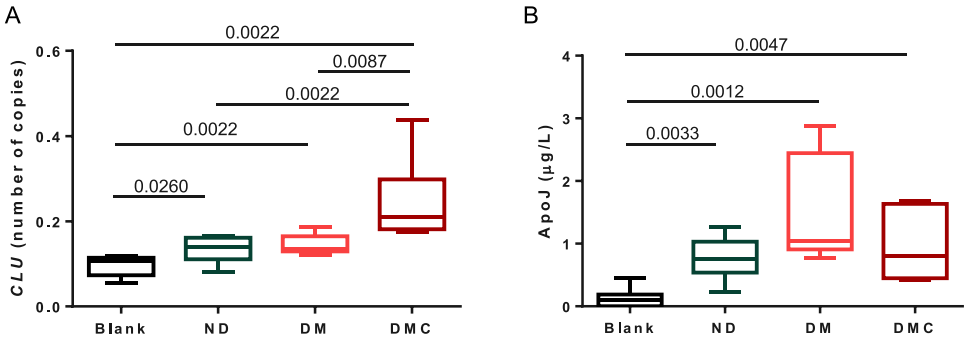

Fig. 9. Apolipoprotein J (apoJ, clusterin CLU) expression (A) and release (B) induced by EAT secretomes in AC16 cardiomyocytes. For expression analysis, AC16 cells were incubated with the secretome (diluted 1/20) of EAT from nondiabetic patients (ND), diabetic patients (DM), and patients with diabetes plus coronary heart disease (DM-C) ($n = 6$ for each group) for 4 h. Afterwards, RNA was extracted from the cells and RT-PCR was performed from cDNA, as described in Methods. The expression of CLU was relativized to β-actin expression and expressed as number fold vs. blank (basal expression of AC16 cells not incubated with secretome). For protein release, cells were incubated with the secretome of EAT from the ND, DM, and DM-C ($n = 7$) patients for 24 h. Afterwards, apoJ release was evaluated in the supernatant by ELISA, as described in the Methods. Data are expressed as mean ± SD. Horizontal lines indicate statistically significant differences.

pathway [25,27,70,71]. In addition, apoJ improves myocardial performance following myocardial infarction [29]. Due to these protective functions, we analyzed the role of apoJ in preventing the inflammatory and cytotoxic effects of the secretomes of the three groups analyzed. The role of HDL, a lipoprotein with well-established anti-inflammatory and antiapoptotic properties and the main carrier of apoJ in blood, was also studied. In our study, the exogenous addition to cardiomyocytes of apoJ or HDL inhibited the inflammation promoted by EAT secretomes from the DM patients. Both apoJ and HDL were added at similar concentrations to those detected in blood. In our experimental conditions, apoJ was more efficient than HDL in preventing cytokine release, and especially IL6 release, which was not inhibited by HDL. Regarding apoptosis/cytotoxicity, both apoJ and HDL inhibited the necrotic effect induced by the DM and DM-C secretomes. In addition, secretomes from all patients induced the expression and release of apoJ by AC16 cardiomyocytes, probably in response to cell damage. Accordingly, the highest *CLU* expression was induced by the secretomes from the DM-C samples, which also promoted the highest necrotic effect on cardiomyocytes. However, perhaps due to the high toxicity elicited by the secretomes from the DM-C samples, the release of apoJ was lower

(although not statistically significant) to that promoted by the secretomes from DM patients.

It is well established that HDL exerts cytoprotective effects against inflammatory and apoptotic stimuli, such as PA, in numerous cell types [72,73]. Although HDL transports numerous molecules with cytoprotective potential, including lipids, apolipoproteins, and enzymatic activities, some of the cardioprotective properties of HDL have been ascribed to its apoJ content [74]. In the context of myocardial damage, the role of apoJ seems more relevant than that of HDL, since the myocardial tissue itself also expresses and secretes apoJ, while HDL would come from the blood circulation and presumably be scarce in this environment.

Interestingly, diabetic patients have higher apoJ concentrations in plasma than nondiabetic ones [75], but the apoJ cargo in HDL is lower [74], which would compromise the antiapoptotic and anti-inflammatory capacity of HDL in these patients. In our study, we observed that EAT from the DM patients expressed and released more apoJ than that from the nondiabetic ones, which could be considered a protective response to attenuate the cytotoxic and inflammatory effects of their secretomes. The lack of increased apoJ release from the EAT of the DM-C patients could be interpreted as a loss of the protective capacity of EAT mediated by apoJ in the presence of coronary heart disease.

Our study has several limitations. First, as a proof-of-concept study, the number of samples analyzed was relatively low, and further studies with a larger number of patients should be conducted to confirm our findings. Second, the molecular mechanisms underlying the protective effect of apoJ were not investigated, and they also require further investigation.

In summary, the results showed that EAT from diabetic patients can play a pivotal role in heart dysfunction by promoting inflammatory and cytotoxic effects on cardiomyocytes. Lipid mediators released by EAT likely contribute to both actions, with some specific Cer and NEFA species presumably involved. The release of apoJ was increased in DM-EAT, probably as a compensatory response, and may partly counteract such effects. In turn, the secretomes of diabetic patients also promoted apoJ release by cardiomyocytes, probably with the purpose to minimize their harmful effects. Interestingly, the protective effect of apoJ could be compromised in the presence of coronary heart disease, a situation in which the cytotoxic action of secretomes is stronger. These findings are summarized in Fig. 10. Upcoming investigations evaluating the effect of alterations in other molecules, including proteins and micro-RNA, within the secretomes of diabetic patients with or without coronary disease are warranted.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committees of the Hospital de la Santa Creu i Sant Pau (IIBSP-REL-2017–27). All subjects gave written informed consent before participating in the study.

Consent for publication

Written informed consent to participate in the study and publish results in medical journals was obtained from patients.

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CRediT authorship contribution statement

Inka Miñambres: Writing – review & editing, Methodology, Investigation, Formal analysis. **Pedro Gil-Millan:** Methodology, Investigation, Formal analysis. **José Rives:** Writing – original draft, Methodology, Investigation, Formal analysis. **Núria Puig:** Writing – original draft, Methodology, Investigation, Formal analysis. **Mar Hernández-Guillamon:** Methodology, Investigation. **Anna Bonaterra-Pastra:** Methodology, Investigation. **Manel Tauron:** Methodology,

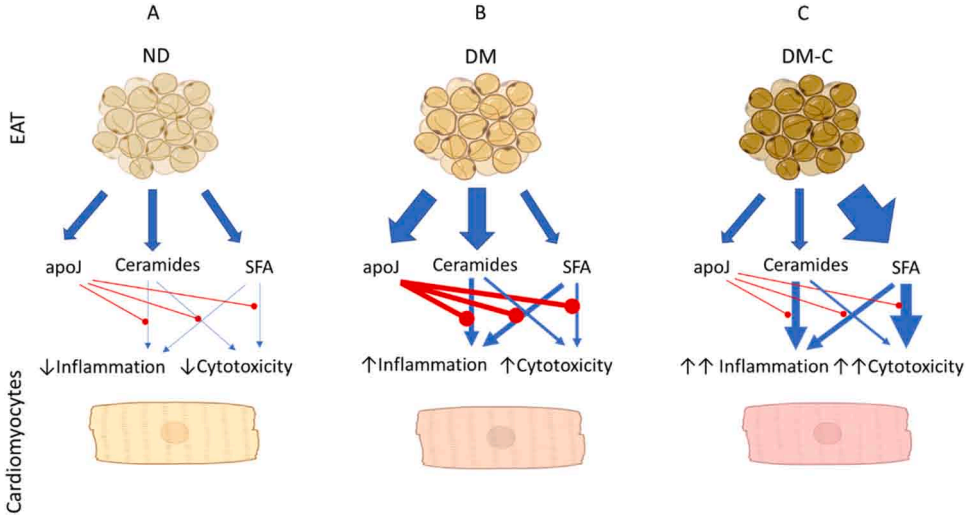

Fig. 10. Summary of the effect of EAT secretomes from nondiabetic (ND), diabetic (DM), and patients with diabetes plus coronary disease (DM-C) on cardiomyocyte function and the putative protective effect of apoJ. (A) The secretomes from the ND patients release moderate amounts of inflammatory/cytotoxic species of ceramides and saturated fatty acids (SFA) and consequently induce a low level of inflammation and cytotoxicity in cardiomyocytes. In this situation, the expression of apoJ by EAT is low. (B) In contrast, the secretomes from the DM patients release an increased concentration of toxic species of ceramides, increasing the development of inflammatory and cytotoxic phenomena. However, the expression of apoJ by the EAT is high and could partially counteract the deleterious effects of the DM secretomes. (C) The co-existence of diabetes and coronary disease increases the release of saturated fatty acids, which exacerbates inflammation and cytotoxicity in cardiomyocytes. This effect is even worse because the modulatory effect of apoJ is lost due to decreased expression. Red lines indicate the inhibitory effect of apoJ.

Investigation. **Antonino Ginel:** Methodology, Investigation. **Jose Luis Sanchez-Quesada:** Writing – review & editing, Writing – original draft, Resources, Formal analysis, Conceptualization. **Antonio Pérez:** Writing – review & editing, Resources, Formal analysis, Conceptualization. **Sonia Benitez:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that no generative AI and AI-assisted technologies have been used in the writing process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116779.

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