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Role of the interaction between endoplasmic reticulum and mitochondria in endothelial dysfunction induced by human microparticles

Zainab Safiedeen

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Thèse de Doctorat

Zainab SAFIEDEEN

THESE EN COTUTELLE

*Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université d'Angers
sous le sceau de l'Université Bretagne Loire*

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Rôle de l'interaction entre le réticulum endoplasmique et les mitochondries dans la dysfonction endothéliale induite par des microparticules humaines

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Table of Materials

Acknowledgements	3
Publications & communications	7
Abbreviations	8
List of Figures	10
List of Tables	10
Introduction	11
A- METABOLIC SYNDROME	11
a) Definition & clinical outcomes	11
b) Metabolic syndrome (MetS) criteria	13
B- MICROPARTICLES	15
a) Definition of MPs.....	15
b) Characteristics of MPs	17
1. Structure, origine and types.....	17
2. Composition	19
3. Mechanisms of MP production	19
4. Mechanisms of MP interaction with target cells.....	20
c) Role of MetS in the liberation of MPs	22
C- CARDIOVASCULAR DISEASES AS CONSEQUENCES OF METS	22
a) Epidemiology	22
b) CVDs and MetS	23
c) Endothelial dysfunction	23
1. Definition	24
2. Endothelial dysfunction and MetS	24
3. MPs: players in endothelial dysfunction	25
4. Neutral sphingomyelinases: another worker in endothelial dysfunction	26
D- ENDOPLASMIC RETICULUM STRESS	27
a) Endoplasmic reticulum (ER) histology and function.....	27
b) ER stress: the unfolded protein response	27
c) Endoplasmic reticulum stress: its role in diseases and a novel target for therapy	30
d) ER stress is involved in MetS and endothelial dysfunction.....	32
e) ER stress partners in crime: mitochondria and oxidative stress.....	32

1. Mitochondria histology and function	32
2. Mitochondria and mitochondrial reactive oxygen species as mediators of metabolic alterations and endothelial dysfunction.....	33
3. ER and mitochondrial interaction	35
4. ER and oxidative stress dialogue	36
Aim of the study.....	37
Article	38
Discussion & Conclusion	39
Review	43
Publication	44
References	45

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To my family
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ABBREVIATIONS

4-PBA: 4-phenylbutyrate

AACE: American Association of Clinical Endocrinologists

ATF4: activating transcription factor 4

ATF6: activating transcription factor 6

BH4: Tetrahydrobiopterin

BIP: immunoglobulin heavy chain-binding protein

BP: blood pressure

CEACAM: carcinoembryonic antigen-related cell adhesion molecule

CHOP: C/EBP homologous protein

cROS: cytosolic ROS

CRP: C-reactive protein

CVDs: cardiovascular diseases

EGIR: European Group for Study of IR

eIF2 α : eukaryotic initiation factor 2 α

EMPs: Endothelial-derived MPs

eNOS: endothelial NO synthase

ER: endoplasmic reticulum

ERAD: ER-associated protein degradation

EVs: extracellular vesicles

GRP75: glucose regulated protein 75

GRP78: glucose regulated protein

HDL: high-density lipoprotein

HDLc: HDL cholesterol

ICAM-1: intercellular adhesion molecule

IDF: International Diabetes Federation

ILs: interleukins

IP3R: inositol triphosphate receptor

IRE1: inositol requiring kinase 1

LDL: low density lipoprotein

LDLR: low-density lipoprotein receptor

LMPs: Leukocyte-derived MPs

Mac-1: macrophage antigen-1

MAMs: mitochondria-associated membranes
MCU: mitochondrial Ca²⁺ uniport
MetS MPs: metabolic syndrome microparticles
MetS: metabolic syndrome
Mfn1: mitofusin-1
Mfn2: mitofusin-2
MMPs: Monocyte-derived MPs
MPs: microparticles
mROS: mitochondrial ROS
NCEP ATPIII: National Cholesterol Education Program Adult Treatment Expert Panel III
NO: nitric oxide
NSMases: neutral sphingomyelinases
OXPHOS: oxidative phosphorylation
PECAM: platelet endothelial cell adhesion molecule
PERK: protein kinase (PKR)-like endoplasmic reticulum kinase
PMPs: platelet-derived MPs
PS: phosphatidylserine
ROS: reactive oxygen species
SMases: sphingomyelinases
T2DM: type 2 diabetes mellitus
TNF- α : tumor necrosis factor alpha
TRAIL: tumor necrosis factor (TNF)-related apoptosis inducing ligand
Tudca: tauroursodeoxycholic acid
UPR: unfolded protein response
VDAC: voltage-dependent anion channel
WHO: The World Health Organization
XBPI: X boxbinding protein 1

LIST OF FIGURES

Figure 1. Metabolic syndrome criteria leading to cardiovascular diseases.	14
Figure 2. Biogenesis of extracellular vesicles and content of MPs.	16
Figure 3. Schematic representation of the panel of molecules transmitted by MPs.	19
Figure 4. Mechanisms of MPs formation and their mode of interaction with target cell.	21
Figure 5. Schematic representation of MPs- induced endothelial dysfunction.	26
Figure 6. Overview of the three signaling branches of the ER stress response, the UPR.	29
Figure 7. Uncoupling the endothelial NO synthase (eNOS); the mechanism through which ROS participates in endothelial dysfunction and subsequent vascular diseases.	34
Figure 8. Mitochondria-associated endoplasmic reticulum complex.	35
Figure 9. The pathways taken by apoptotic T MPs and MetS MPs in inducing endothelial dysfunction.	42

LIST OF TABLES

Table 1. Criteria set out for the diagnosis of MetS according to a number of influential associations.	12
Table 2. Types of microparticles, composition, and role in disease progression.	18
Table 3. Human diseases linked to ER stress.	30
Table 4. Compounds with potency to ameliorate ER stress.	31

INTRODUCTION

A- METABOLIC SYNDROME

a) Definition & clinical outcomes

Metabolic syndrome (MetS) is a rising and a master clinical concern emerging worldwide nowadays, due to the easy life style, lack of exercise and increasing obesity. For this, it stood out as a concept rather than a diagnosis ⁽¹⁾. It was first defined as a cluster of physiological risk factors that are present in higher deal than predictable conditions ⁽²⁾. In 1975, Haller and Hanefeld characterized the term MetS as the multiplex risk factors, when combined all together culminate in different outcomes including type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVDs), and consequently increasing the rate of mortality ⁽³⁾. Later, MetS was defined as a unified group of physiological, biochemical, clinical and metabolic factors that directly link to increased risk of atherosclerotic CVD and all reasons of mortality. Also, it was defined as a state of chronic low grade inflammation that emerges as a result of environmental and genetic factors ⁽⁴⁾.

The World Health Organization (WHO) ⁽⁵⁾, the European Group for Study of IR (EGIR) ⁽⁶⁾, the National Cholesterol Education Program Adult Treatment Expert Panel III (NCEP ATP III) ⁽⁷⁾, the American Association of Clinical Endocrinologists (AACE) ⁽⁸⁾, and the International Diabetes Federation (IDF) ⁽⁹⁾, identified components of the MetS that relate to increased consequential risk of CVD. As detailed in Table 1, each of these groups has developed their own (granted, typically overlapping) criteria for defining MetS, with regard to component's priority and thresholds for each of the variables of MetS ⁽¹⁰⁾ in order to accurately identify people at a higher than average risk of developing CVDs.

Table 1. Criteria set out for the diagnosis of MetS according to a number of influential associations

	<i>WHO</i>	<i>EGIR</i>	<i>NCEP: ATPIII</i>	<i>AACE</i>	<i>IDF</i>
Priority component	High insulin level+ Two of the following:	High fasting insulin concentrations /insulin resistance + Two of the following:	Any three of the following:	Impaired glucose Tolerance + Two of the following:	Central obesity = WC (ethnicity and gender specific) + Two of the following:
Elevated waist circumference	> 37 in	≥ 37 in males ≥32 in females	> 40 in males >35 in females		≥ 37 in males ≥32 in females
Elevated triglyceride	>150 mg dL ⁻¹	>78 mg dL ⁻¹	≥150 mg dL ⁻¹	≥150 mg dL ⁻¹	≥150 mg dL ⁻¹
Reduced HDL-C	<35 mg dL ⁻¹ in males <39 mg dL ⁻¹ in females	<1 mg dL ⁻¹	<40 mg dL ⁻¹ in males <50 mg dL ⁻¹ in females	<40 mg dL ⁻¹ in males <50 mg dL ⁻¹ in females	<40 mg dL ⁻¹ in males <50 mg dL ⁻¹ in females
Elevated blood pressure	≥ 140/90 mm Hg	≥ 140/90 mm Hg	≥ 130/85 mm Hg	≥ 130/85 mm Hg	≥ 130/85 mm Hg
Elevated fasting glucose	≥ 6.1 mmol L ⁻¹ (¹¹)	≥6.1 mmol L ⁻¹	≥ 6.1 mmol L ⁻¹		≥5.6 mmol L ⁻¹ or T2DM

HDL: high-density lipoprotein, BP: blood pressure, T2DM: type 2 diabetes mellitus. ⁽³⁾

b) Metabolic syndrome (MetS) criteria

In addition to the criteria proposed by the different organizations (Table 1), other additional factors such as pro-inflammatory (high levels of C-reactive protein (CRP)) and prothrombic (enhanced coagulation, platelet dysfunction) states, physical inactivity, smoking, and aging characterize MetS. All of which are considered underlying, major, emerging risk factors for CVD⁽¹²⁾. However, only five components are used for defining metabolic subjects. Therefore, MetS is defined as a condition where an individual presents at least 3 of the following criteria (i) abdominal obesity (precisely waist circumference), (ii) increased fasting glucose, (iii) high blood pressure, (iv) increased plasma triglyceride, and (v) reduced high-density lipoprotein (HDL) cholesterol concentrations⁽¹³⁾ (Figure 1).

Obesity: The prevalence of MetS is directly correlated with the development of obesity⁽¹⁴⁾. Evidence refers that MetS starts with excess central adiposity⁽¹⁵⁾. It is mostly believed that it is a result of excess triglycerides in adipose tissue⁽¹⁶⁾, which is a result of a major nutrient consumption than that demanded for normal metabolism⁽¹⁷⁾. A person is considered obese when the body mass index is higher than 30 kg/m²⁽¹⁸⁾.

Abdominal obesity is the strongest form associated with MetS. It is expressed clinically as increased waist circumference⁽¹²⁾. Increased abdominal fat mass also known as “apple-shaped” model showed to be directly linked to MetS development. More than that, intra-abdominal fat is well known nowadays as an active endocrine organ responsible for secreting a wide spectrum of cytokines like interleukins (ILs), tumor necrosis factor alpha (TNF- α), all of which that act on increasing metabolic disorders⁽¹⁹⁾. Interestingly, obesity is considered in all MetS definitions, for example, most obese people have postprandial hyperinsulinemia and relatively low insulin sensitivity⁽²⁰⁾.

Hyperglycemia: Most persons with the MetS have high levels of plasma glucose⁽²¹⁾. The primary cause of hyperglycemia in patients with MetS is insulin resistance⁽²²⁾, characterized by high plasma insulin concentration that is unable to decrease plasma glucose⁽²³⁾. The factors participating to insulin resistance are complex, however, high concentrations of free fatty acid represents a key event. This also explains the interaction between different MetS criteria⁽¹⁹⁾. Moreover, it is considered as the combining mechanism underlying the clustering of metabolic abnormalities⁽²⁴⁾, and majority of persons with MetS exhibit insulin resistance⁽²³⁾. For this, ATP III considers insulin resistance as one of the MetS components⁽¹²⁾.

Dyslipidemia: Dyslipidemia is characterized by high levels of triglycerides⁽¹⁹⁾ and low concentrations of high density lipoprotein (HDL) cholesterol⁽²⁵⁾. Also, MetS cases are correlated with atherogenic dyslipidemia. High levels of low density lipoprotein (LDL)

represent the major ingredient of atherogenic dyslipidemia and the primary cause of atherosclerotic cardiovascular diseases ⁽²⁶⁾. These two features correlate with weight gain and increased myocardial risk ⁽¹⁹⁾.

Hypertension: Another criterion of MetS is high blood pressure. Increased blood pressure is usually associated with obesity and high calorie uptake. Several mechanisms have been proposed to explain the relationship between elevated blood pressure and obesity, such as release of angiotensinogen from adipose tissue ⁽²⁷⁾. In addition, patients with hypertension have been shown to be resistant to insulin-stimulated glucose uptake and to be both hyperinsulinemic and hypertriglyceridemic compared with matched normotensive control groups ⁽²⁸⁾.

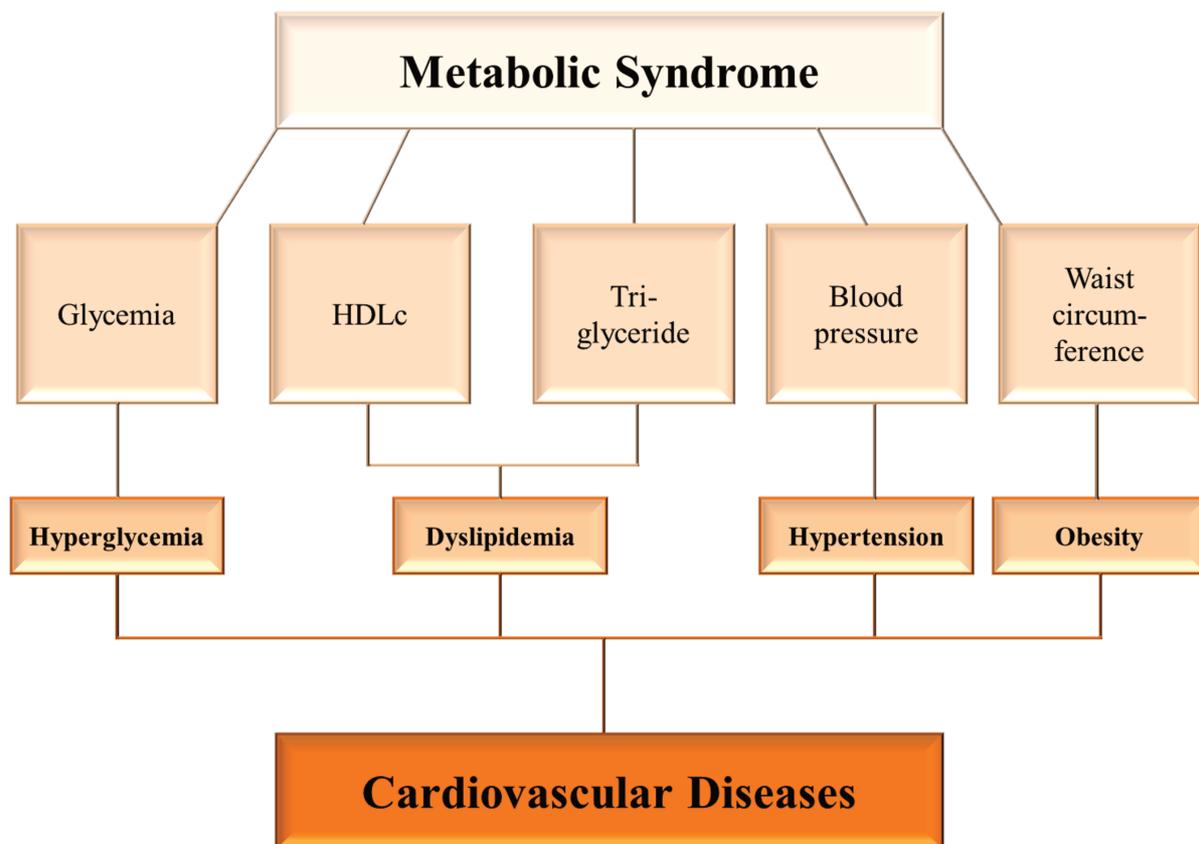


Figure 1. Metabolic syndrome criteria leading to cardiovascular diseases. Hyperglycemia, decreased HDL cholesterol (HDLc) and increased triglyceride levels resulting in dyslipidemia, high blood pressure leading to hypertension and raised waist circumference present in obesity, result in cardiovascular diseases.

Recently, new elements emerged as effectors in metabolic and cardiovascular pathologies. Among these are the microparticles (MPs).

B- MICROPARTICLES

Cell-cell cross-talk is an essential element of development, homeostasis maintenance and cell defense. Far from the classical signaling through cell-cell contact and soluble factors, intracellular signaling via extracellular vesicles (EVs) emerges as a mode of communication with an ability to deliver various messages. EVs comprise exosomes, MPs and apoptotic body.⁽²⁹⁾ Among these types of EVs, only the effects of MPs on MetS have been described in the literature.

a) Definition of MPs

MPs are small membrane vesicles, heterogeneous in size with less than 1 μm in diameter, released from cells in response to activation or apoptosis⁽³⁰⁾. These vesicles are produced from the blebbing of the plasma membrane⁽³¹⁾.

MPs differ from exosomes and apoptotic bodies by the mechanism implicated in their formation and by their size and composition. In fact, exosomes are assumed to be a homogenous population of vesicles of 0.02-0.1 μm size. They are formed within the endosome by invagination of the limiting membranes, resulting in the formation of multivesicular bodies. Subsequently, multivesicular bodies fuse with the plasma membrane and release exosomes into the extracellular environment⁽³²⁾. Apoptotic bodies are large vesicles (0.5 - 4 μm), formed during programmed cell death following cell fragmentation. These vesicles have a high content of cytoplasmic and nuclear elements (proteins and nucleic acids)⁽³³⁾ (Fig 2).

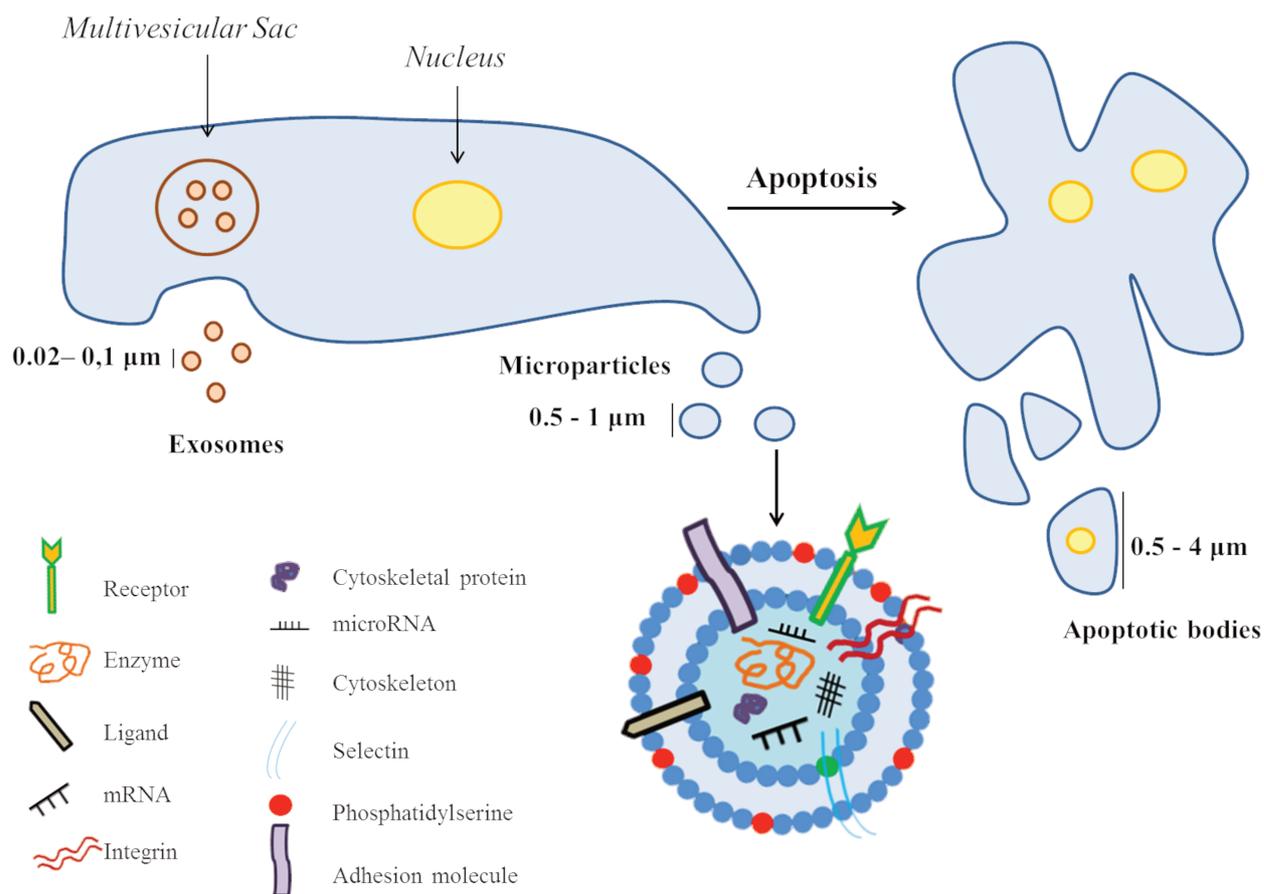


Figure 2. Biogenesis of extracellular vesicles and content of MPs.

(Modified from Akers et al, 2013⁽³³⁾, and Gaceb et al, 2014⁽³⁴⁾). The exosomes (0.02 to 0.1 μm) are formed within the cell and transported to the outside using a multivesicular sac. The apoptotic bodies are large vesicles (0.5 to 4 μm) detached from the cell during the terminal phase of apoptosis. MPs (0.5 to 1 μm) produced by budding from the plasma membrane, carry on their surface phosphatidylserine, adhesion molecules, selectins, integrins, ligands, and receptors; whereas their inside is rich in cytoskeletal proteins, enzymes, mRNA and microRNA.

MPs which were initially inspected as cellular dust, are considered nowadays new modes of cellular communication, biomarkers for predicting diseases and potential therapeutic targets. Particularly, several lines of data support the hypothesis that MPs contribute to vascular homeostasis and the pathogenesis of CVD, including inflammation and vascular dysfunction⁽³¹⁾.

b) Characteristics of MPs

1. Structure, origine and types

MPs emerging from the cell share the same cytoplasm, nucleic acid, and cytoskeleton residues that the mother cell. They are surrounded by a phospholipid bilayer associated with proteins such as receptors, ligands, selectins and integrins. As a consequence of cell activation or apoptosis, which result in MP formation, the negatively charged phospholipids such as the phosphatidylserine (PS) becomes in the outer leaflet of the membrane, resulting in MPs bearing PS in their membranes ⁽³⁵⁾.

Based on their specific surface proteins, it was found that MPs are released from cells of vascular wall (such as endothelial cells and smooth muscle cells) or from circulating cells (such as platelets, erythrocytes, T and B cells, monocytes) and tumor cells ⁽³⁶⁾. However, platelet-derived MPs (PMPs) represent ~70-90% of circulating MPs ⁽³⁰⁾. In addition, MPs can be found in various biofluids (blood, urine, ...) ⁽³⁷⁾.

The types of MPs and their role in disease progression are summarized in Table 2.

Table 2. Types of microparticles, composition, and role in disease progression.

Types of microparticles ⁽³⁸⁾	Composition ^{(39) (40)}	Role in disease progression ⁽³⁸⁾
Platelet-derived MPs (PMPs)	Phosphatidylcholine (60%), sphingomyelin, phosphatidylethanolamine, phosphatidylserine, P-selectin, glycoprotein 53, proteins of chemokines.	Coagulation, inflammatory processes, thrombosis, and tumor progression
Endothelial-derived MPs (EMPs)	Oxidized phospholipids, E-selectin, proteins of metabolic enzymes and fusion processes, cytoskeleton-associated proteins	Endothelial dysfunction, tumor growth, and increased oxidative stress
Monocyte-derived MPs (MMPs)	Phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysophospholipids and small amounts of PS	Endothelial dysfunction, sepsis, and vascular inflammation
Leukocyte-derived MPs (LMPs)	L-selectin ⁽⁴¹⁾ , sphingomyelin, PS harbor cell-specific antigens such as CD11b, CD66b, and CD15, bioactive proteins such as interleukin 1 β , CD40 ligand, major histocompatibility complex class I and II, and intercellular adhesion molecule (ICAM)-1, matrix metalloproteinase ⁽⁴²⁾	Endothelial dysfunction, obstructive sleep apnoea, diabetes, and hypertension ⁽⁴³⁾

2. Composition

Proteome analysis revealed that the spectrum of proteins found in the MPs *in vitro* generated depends partly on the stimulus used to induce their formation ⁽⁴⁴⁾. Currently, it is well known that the number and composition of MPs vary greatly according to the cellular origin, and also, as the stimulus involved in their formation (Figure 3). Briefly, MPs harbor (i) cholesterol and phospholipids including lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, lysophosphatidylserine, PS, lysophosphatidylethanolamine, phosphatidylethanolamine and phosphatidylinositol ⁽⁴⁵⁾; (ii) surface markers, proteins, and enzymes: for example PMPs can expose activation markers such as P-selectin when MPs derived from platelets, CD8 when derived from lymphocytes, and CD146 when generated from endothelial cells (Diamant et al), and (iii) nucleic acids ⁽⁴⁶⁾.

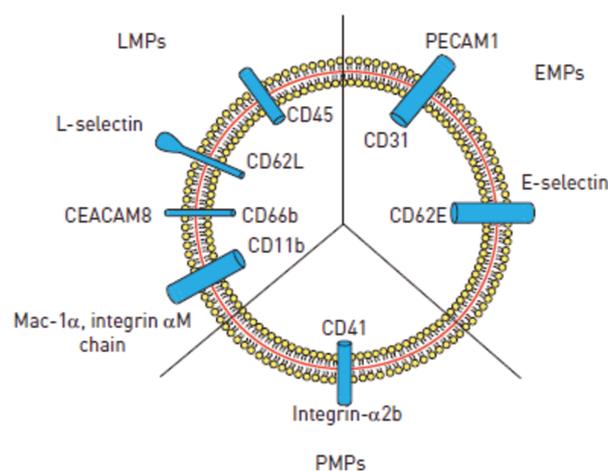


Figure 3. Schematic representation of the panel of molecules transmitted by MPs.

(From Trzeplur et al, 2014 ⁽⁴³⁾) LMP: leukocyte-derived microparticle; EMP: endothelium-derived microparticle; PMP: platelet-derived microparticle; CEACAM: carcinoembryonic antigen-related cell adhesion molecule; Mac-1: macrophage antigen-1; PECAM: platelet endothelial cell adhesion molecule.

3. Mechanisms of MP production

MP formation and shedding demand reconstitution of cell membrane phospholipid structure, with the outer leaflet subjected to PS, modification of cell architecture, and disruption of cytoskeleton organization ⁽⁴⁷⁾. More precisely, MPs are produced after chemical or physical cell activation, by different agonists such as thrombin or collagen, which in turn launches a sustained elevation of cytosolic Ca^{2+} influx, that activates calpain or leads to kinase activation

(protein kinase C, tyrosine protein kinases) and phosphatase inhibition, resulting eventually in cytoskeleton blebbing⁽⁴⁸⁾. During apoptosis, membrane blebbing and MP formation results from different signaling apoptotic pathways such as ROCK1 and caspase 3, and the tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) and its receptor TRAIL-R2. Moreover, their quantity can vary based on their mode of formation (Fig 4)⁽⁴⁹⁾. Moreover, oxidative stress and inflammation are possible key factors in promoting MP generation⁽⁵⁰⁾.

4. Mechanisms of MP interaction with target cells

MPs can communicate with target cells by different mechanisms. 1- By interacting with the ligands present on the surface of target cells, MPs activate cascade signaling. For example, we have previously shown that MPs isolated from MetS patients bearing Fas ligand interacts with Fas receptor present on vascular smooth muscle cells causing vascular hyporeactivity⁽⁵¹⁾; 2- MPs can transfer part of their components without fusion with target cells. Indeed, it has been shown that the chemokine receptor CCR5 was transferred to mononuclear CCR5⁺ cells after their incubation with MPs bearing the concerned receptor⁽⁵²⁾; 3- MPs can transfer proteins, mRNA, miRNA, and bioactive lipids by fusing with target cells. For example, the fusion of MPs derived from mature macrophages with the monocyte plasma membrane permits their differentiation into macrophages through the transfer of miR-223 carried by the MPs⁽⁵³⁾; 4- Internalization of MPs within the target cells. We have shown that internalization of lymphocytic T-MPs protects endothelial cells from apoptosis, partially by decreasing cytoplasmic reactive oxygen species (ROS) and by transferring antioxidant enzymes such as the superoxide dismutase⁽⁵⁴⁾ (Fig 4).

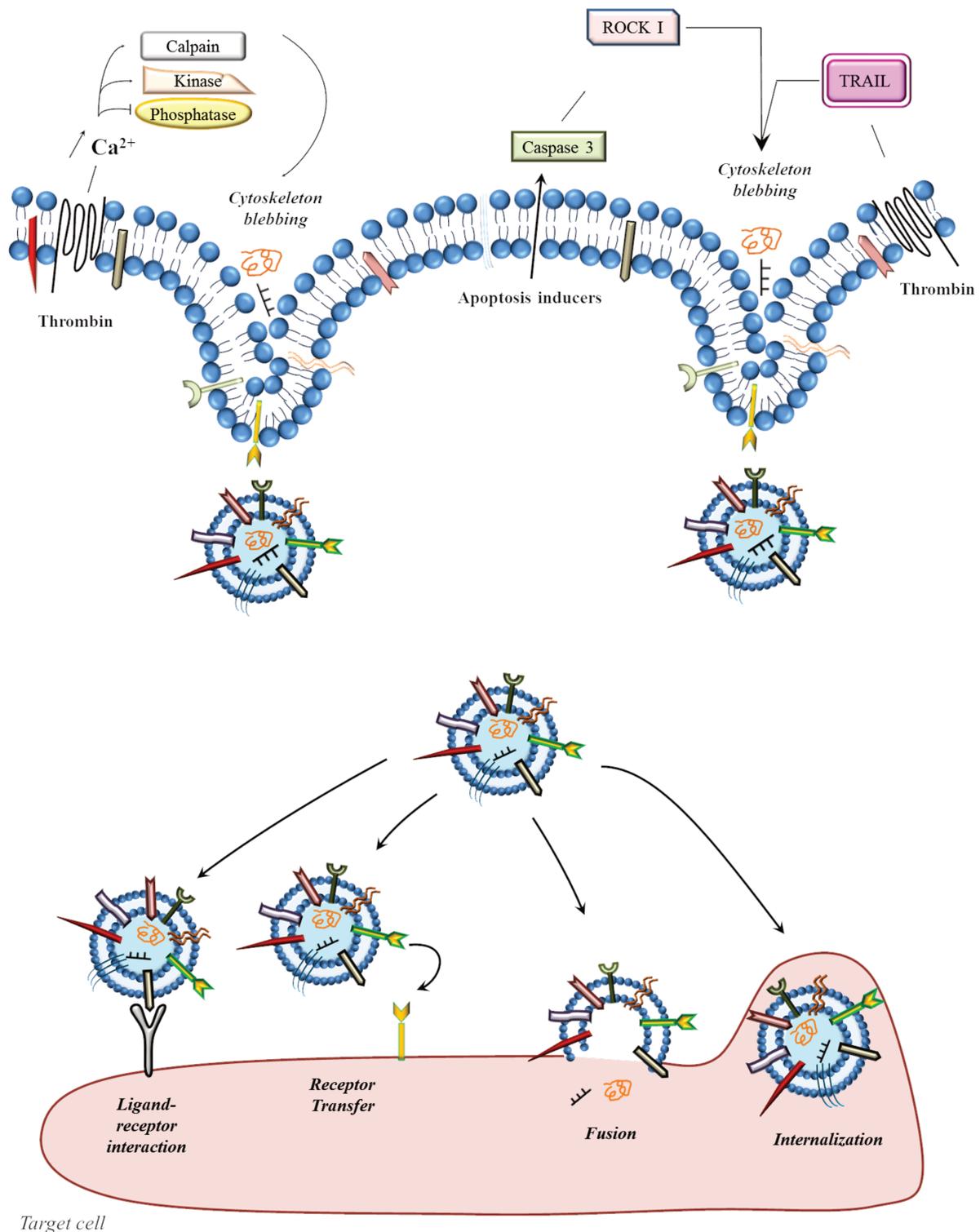


Figure 4. Mechanisms of MPs formation and their mode of interaction with target cell. (Modified from Tual-Chalot et al, 2011 ⁽⁵⁵⁾). MP formation results from cytoskeleton blebbing due to cellular activation or apoptosis. Once they are produced, MPs interact with target cells either through ligand-receptor interaction, transfer of receptor, fusion or complete internalization of MPs. ROCKI, Rho-associated protein kinase1; TRAIL, tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL).

c) Role of MetS in the liberation of MPs

Previous studies have shown an increase in the levels of circulating MPs in MetS subjects in comparison to healthy ones. Indeed, levels of MPs from pro-coagulant (Annexin V⁺), platelets, erythrocytes and endothelial cells are increased in individuals with MetS *versus* healthy subjects ⁽⁵⁶⁾. Arteaga and colleagues ⁽⁵⁷⁾ have reported marked elevation of EMPs levels in patients with MetS compared to controls. Also, it has been shown that the postprandial state after a high-fat meal induces the release of EMPs and that the magnitude of postprandial triglyceridemia correlates with the postprandial increase in circulating EMP levels ⁽⁵⁸⁾. In addition, Chironi and colleagues ⁽⁵⁹⁾ have shown that, in patients with MetS, leukocyte-derived MP levels are higher than that in those free of such syndrome. Moreover, in the overall study population, leukocyte-derived MP levels increase gradually in parallel with the number of components of MetS. Finally, Helal et al. ⁽⁵⁰⁾, revealed the association of MPs with the components of the MetS, and their deleterious impact on atherothrombosis. Indeed, the relationship between MP subpopulations and hyperglycemia, hypertension, the extent of lipidemia and the level of HDL cholesterol have been reported mainly in advanced stages of cardiovascular disorders. Therefore, it could be speculated that MPs are possible biomarkers for the identification of those subjects with MetS and an elevated risk of developing cardiovascular complications ⁽⁵⁰⁾.

C- CARDIOVASCULAR DISEASES AS CONSEQUENCES OF METS

a) Epidemiology

Cardiovascular pathologies comprise stroke, arterial fibrillation, sudden cardiac arrest, coronary heart disease, and heart failure ⁽⁶⁰⁾. All of which remain the major cause of death among Europeans and worldwide (29.6% of all deaths), and despite recent decrease in mortality rates in many countries, it is still responsible for over 4 million deaths per year, close to half of all deaths in Europe. More than all communicable, maternal, neonatal and nutritional disorders double the number of deaths caused by cancers. Referring to an epidemiological overview done on 2014, which based on a number of European and international data sources, CVDs continue to cause a much greater mortality burden among Europeans than any other disease. Overall, CVDs caused 51% of deaths among women and

international data sources, CVDs continue to cause a much greater mortality burden among Europeans than any other disease. Overall, CVDs caused 51% of deaths among women and 42% among men in the last year of data, compared with 19 and 23%, respectively, for all cancers ⁽⁶¹⁾. A statistical update done by the American Heart Association in 2015 showed that CVDs prevalence was higher in children and young adults, in comparison to middle-aged and older adults. These risk factors were referred to the poor lifestyle behaviors, lifestyle-related risk factors (tobacco, high fat diets, ...) and physical inactivity ⁽⁶⁰⁾.

b) CVDs and MetS

MetS is a major cause of CVDs. Several studies have shown that the risk of CVDs is correlated with the number of MetS components, as the risk increases with the increase in the number of MetS components ⁽¹⁹⁾. Also, patients with coronary heart disease or those who have experienced a stroke usually display more than one major metabolic criterion ⁽⁶⁰⁾. This is agreement with the fact that the rate of ischemic stroke is higher in MetS patients compared to non-metabolic ones ⁽⁶²⁾. Moreover, the Third National Health and Nutrition Examination Survey has reported that the increased risk of myocardial infarction is correlated with the presence of MetS ⁽⁶³⁾. Lately, it has been estimated that increased vasoconstrictive substances and increased prothrombotic adipokines takes charge of increased CVD incidence, both of which are associated with MetS ⁽²³⁾. Also, the assembly of hypertension and obesity is also known to cause higher rate of morbidity and mortality associated with CVDs ⁽⁶⁴⁾. The atherogenic profile which consists of increased triglycerides and LDL, and decreased HDL, increases the risk of CVDs. Furthermore, diabetic hyperlipidemia or hyperglycemia accelerates atherogenesis ⁽⁶⁵⁾. Finally, components of MetS may be involved, directly or indirectly, in endothelial dysfunction which is associated with atherosclerosis initiation and progression ⁽⁶⁶⁾, a marker of cardiovascular outcomes ⁽⁶⁷⁾.

c) Endothelial dysfunction

Several epidemiological studies have reported that endothelial dysfunction is associated with cardiovascular risk factors. Endothelial dysfunction is a systemic phenomenon associated with vascular inflammation, lipid deposition, thrombosis ⁽⁶⁸⁾ and represents an early step in the development of atherosclerosis, a potential lead to CVD development ⁽⁶⁷⁾. Therefore, endothelial dysfunction represents a primary disturbance in cardiovascular events.

1. Definition

Endothelial dysfunction is used to describe abnormal function and alterations of the endothelium, shifting its function toward vasoconstriction instead of vasodilation, pro-inflammatory state and changes in its prothrombotic and proliferative properties ⁽⁶⁹⁾. In other words, endothelial dysfunction results from the imbalance between vasodilatory substances such as nitric oxide (NO), vasoconstrictive ones like endothelin-1 and prothrombotic factors like the plasminogen activator inhibitor-1 ⁽²³⁾. Thus, during endothelial dysfunction, a decrease in NO bioavailability ⁽⁶⁸⁾ an enhancement of ROS liberation, activation of inflammation and alteration of barrier function can be observed ⁽⁷⁰⁾.

2. Endothelial dysfunction and MetS

Individuals with MetS exhibit higher degree of endothelial dysfunction ⁽⁴⁾. Each component of the MetS has been reported to impair endothelial function, and its severity was associated with the number of those components ⁽¹⁵⁾. Ahirwar et al ⁽²³⁾ showed that NO levels in the plasma were lower in MetS patients compared to control ones, reflecting endothelial dysfunction in MetS. Visceral fat tissue drives proatherogenic adipokines production, which in turn contributes in increased oxidative stress and chronic inflammation, both of which affect endothelial function ⁽¹⁵⁾. More precisely, the association of increased visceral obesity and other metabolic perturbations impaired NO biodisposability, causing endothelial dysfunction ⁽⁴⁾. Decreased NO bioavailability due to elevated oxygen species release, may be the major reason of endothelial dysfunction in obesity ⁽¹⁵⁾. On the one hand, insulin resistance may also impair NO bioavailability ⁽⁴⁾. Insulin signaling is important to endothelial cells as it stimulates the production of NO from the endothelium leading to vasodilation. In cases of insulin resistance, the vasoconstrictor endothelin-1 is released from vascular endothelium. This imbalance between vasoconstrictor and vasodilator actions of insulin, under insulin resistance conditions, represents an important factor in the vascular pathophysiology of insulin resistance and endothelial dysfunction ⁽⁷¹⁾. On the other hand, the PI3K/Akt cascade of the insulin signaling pathway is decreased in insulin resistance leading to endothelial damage ⁽⁷²⁾. This emerges insulin resistance as a potential link between MetS and endothelial dysfunction ⁽¹⁵⁾. It has been also demonstrated that some metabolic components cause endothelial dysfunction by boosting several hormones, inflammatory cytokines and molecules ⁽⁴⁾. Furthermore, elevated high sensitive CRP levels, which are a feature of MetS, are associated with reduced basal and stimulated NO release from endothelial cells through various mechanisms such as insulin resistance. Moreover, high leukocyte count may

contribute to endothelial dysfunction, through inflammatory cytokines and cytotoxic products secretion ⁽¹⁵⁾. Also, it has been shown that hypertension is associated with decreased endothelial-dependent relaxation and reduced endothelial NO synthase (eNOS) phosphorylation ⁽⁷³⁾.

3. MPs: players in endothelial dysfunction

Due to their localization in the blood stream, circulating MPs have a major role in interaction with circulating cells or components of the vessel wall including the endothelium, considered as the primary target for cardiovascular risk factor ⁽³¹⁾. Thus, increased levels of circulating MPs released from platelets, leukocytes, erythrocytes and endothelial cells, have been described in diseases associated with cardiovascular complications ⁽⁵⁰⁾. Moreover, EMPs bear molecules able to initiate coagulation, induce monocyte adhesion, activate neutrophils, and affect vasodilation, antithrombotic and antiadhesive properties of the vascular wall ⁽³¹⁾. For all these reasons, MPs can be largely related to pathogenesis of various CVDs mainly launched by endothelial dysfunction ⁽³⁸⁾. Interestingly, increased number of circulating EMPs is found in cases of endothelial cell damage and dysfunction. In addition, EMPs can aggravate endothelial dysfunction. Indeed, EMPs impair endothelium-dependent relaxation and NO release in rat aorta through superoxide anion production ⁽⁷⁴⁾. Apoptotic endothelial MPs originating from damaged endothelial cells are considered as markers of endothelial cell injury and vascular aging ⁽⁷⁵⁾. In addition, circulating MPs, rich in endothelial and platelet surface markers, from patients with acute myocardial infarction cause severe endothelial dysfunction in healthy blood vessels by affecting the endothelial NO transduction pathway, leading to decreased relaxations to acetylcholine in rat aortic rings ⁽⁷⁶⁾.

More than that, *in vitro* apoptotic T lymphocyte-derived MPs, at concentrations that can be reached in circulating blood in pathological disorders, impair endothelium-dependent relaxation in conductance and small resistance arteries in response to agonists and shear pressure, respectively. In addition, these T lymphocyte-derived MPs affect vascular contraction in mouse aorta by acting directly on smooth muscle cells ⁽⁷⁷⁾. Finally, these MPs induce endothelial dysfunction similar to that of circulating MPs, where they decreased NO production and increased ROS production by a mechanism depending on xanthine oxidase activity in endothelial cells. These effects were associated with a reduction of eNOS activity (Fig 5) ⁽⁷⁸⁾. A possible mechanism through which these MPs interact with endothelial cells is through low-density lipoprotein receptor (LDLR)-mediated endocytosis ⁽⁷⁹⁾.

Intravenous injection of MPs collected from MetS subjects promotes vascular hypo-reactivity in mouse aorta. This vascular hypo-reactivity was associated with overproduction of vasodilator mediators, such as NO and prostacyclin, as well as increased ROS. More, precisely, MetS MPs enhance the expression of the NADPH-oxidase subunits such as p47^{phox}. Of particular interest, MetS MPs induce vascular hypo-reactivity through Fas/FasL pathway⁽⁵¹⁾. Finally, Agouni et al⁽⁵⁶⁾ have shown that MPs from MetS patients decrease NO production in cultured endothelial cells. Moreover, these effects were associated with a reduction of eNOS activity, and enhanced nitration of proteins, indicating enhanced levels of ONOO⁻ formation (Fig 5). Also, MPs from MetS patients induce *in vivo* endothelial dysfunction in mouse aorta in response to acetylcholine.

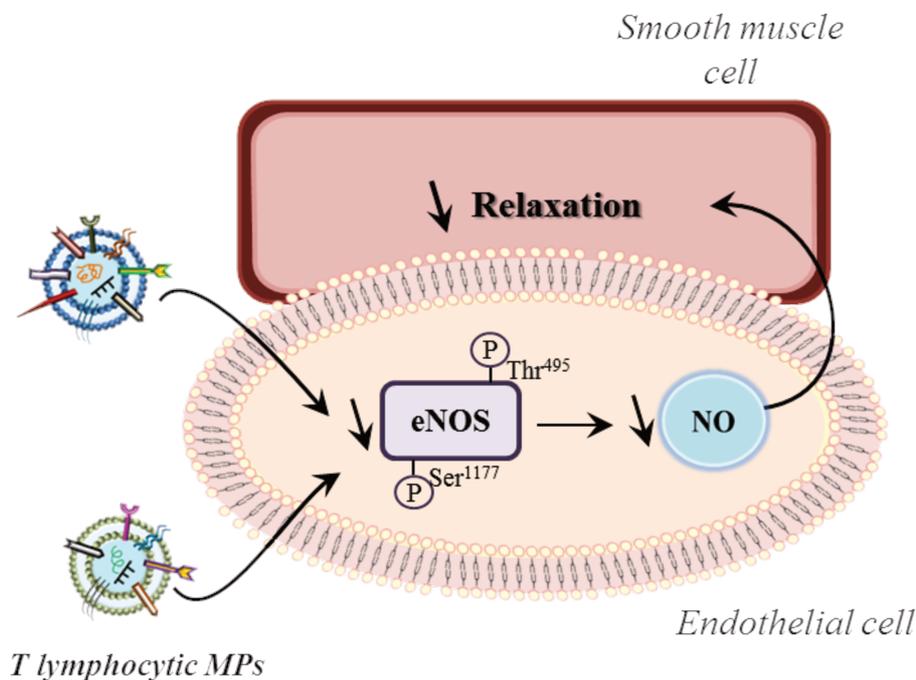


Figure 5. Schematic representation of MPs- induced endothelial dysfunction. Both MetS MPs and T lymphocyte MPs decreased endothelial NO synthase (eNOS) activity, where both MPs increased the ratio of phosphorylated eNOS at the inhibitor and activation sites^(56,78), therefore reducing nitric oxide (NO) bioavailability leading in smooth muscle cell relaxation.

4. Neutral sphingomyelinases: another worker in endothelial dysfunction

Sphingomyelinases (SMases) contribute to cardiovascular physiology. SMases that hydrolyse sphingomyelin, releasing ceramide, and creating a cascade of bioactive lipids, are activated in different cardiovascular system cell types, namely cardiomyocytes, endothelial and vascular smooth muscle cells. Particularly, studies predicted that the membrane neutral

sphingomyelinase (NSMase) plays a critical role in the development of cardiac failure, atherosclerosis, and decline in vasomotion ⁽⁸⁰⁾. ROS production is one mechanism through which NSMases affects the cardiovascular system. NSMase activates hypoxia-induced phosphorylation of NADPH oxidase (p47^{phox}) and ROS formation in vascular smooth muscle cells ⁽⁸¹⁾; with ROS production playing an important role in the development of CVDs ⁽⁸²⁾. Activation of NSMase in arteries from aging rats results in a decrease in eNOS phosphorylation and activation, leading to a loss of vasomotor function ⁽⁸³⁾. Furthermore, supplementation of isolated aortic rings with lipoic acid reversed age-related loss of endothelial glutathione, leading to reduced NSMase activation and ceramide level in the endothelium and improved endothelial NO-dependent vasomotor function ⁽⁸⁴⁾.

D- ENDOPLASMIC RETICULUM STRESS

a) Endoplasmic reticulum (ER) histology and function

The endoplasmic reticulum (ER) is a continuous membrane network in the cytosol ⁽⁸⁵⁾. In eukaryotic cells, the ER exhibits four major physiological functions: (i) it is the site of the synthesis of membrane and secretory proteins; (ii) it is the place of membrane and secretory protein folding into their native conformation, where these nascent proteins undergo post-translational modifications; (iii) the ER is the main site of Ca²⁺ storage which is involved in intracellular cascade signaling; and (iv) the ER membrane is implicated in the biosynthesis of lipids and sterols ⁽⁸⁶⁾. Normal ER functioning can be disrupted when the stream of nascent, unfolded polypeptide chain surpasses the folding capacity of the ER ⁽⁸⁷⁾.

b) ER stress: the unfolded protein response

More than that the disruption of any ER processes, increased oxidative stress, or the activation of SMases through the Fas receptor engagement ⁽⁸⁸⁾, cause ER stress and the subsequent ER stress response termed the unfolded protein response (UPR). The UPR expresses the ER-to-nucleus signaling cascades, to reestablish ER homeostasis. Homeostasis is restored by increasing molecular chaperones expression and ER foldases, or by stimulating phospholipid synthesis and ER-associated protein degradation (ERAD) and autophagy, selectively degrading mRNAs encoding secretory proteins, activating an antioxidant response, and attenuating general translation and transcription of genes encoding secretory proteins. Also, the UPR activates inflammatory and apoptotic signaling pathways ⁽⁸⁹⁾.

The UPR is initiated upon the recognition of unfolded proteins by chaperones, which are normally involved in every aspect of ER quality control ⁽⁹⁰⁾. One of the best characterized ER chaperone proteins is the glucose regulated protein (GRP78) commonly referred to as BIP, the immunoglobulin heavy chain-binding protein ⁽⁹¹⁾. BIP belongs to Hsp70 family ATPase involved in numerous functions, including translocating nascent polypeptides, facilitating de novo protein folding and assembly, targeting misfolded proteins to ERAD machinery, and maintaining Ca²⁺ homeostasis ⁽⁹⁰⁾. BIP binds transiently to newly-synthesized proteins in the ER and more permanently to misfolded, underglycosylated or unassembled proteins avoiding their transport from the ER ⁽⁹²⁾. The prolonged interaction of the folding protein with the chaperone machinery ⁽⁸⁷⁾, sequesters BIP away from the ER luminal domain of the three ER resident transmembrane proteins: the inositol requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), the activating transcription factor 6 α (ATF6 α), leading to their activation, where normally they are kept in an inactive form through their interaction with ER luminal chaperones ⁽⁹³⁾.

Inositol requiring enzyme 1 (IRE1): is a bifunctional enzyme with serine/ threonine protein kinase and endoribonuclease (RNase) activity in its cytosolic domain. It is also a type I transmembrane protein that senses ER stress by its N-terminal luminal domain. Release from suppression by GRP78 triggers its homodimerization and autophosphorylation as part of the activation process. Activated IRE1 cleaves a 26-base fragment from the mRNA encoding X boxbinding protein 1 (XBP1), resulting in spliced XBP1s and translation of a potent transcription factor controlling the expression of genes involved in ERAD and protein folding, as well as other genes implicated in directing the synthesis of phospholipids that are required for the expansion of ER membranes during ER stress ⁽⁹⁴⁾.

PKR-like ER kinase (PERK): is a type I ER transmembrane kinase. When activated by ER stress, PERK homodimerizes and autophosphorylates and then directly phosphorylates Ser51 on the alpha subunit of eukaryotic initiation factor 2 (eIF2 α). Phosphorylated eIF2 α prevents the formation of ribosomal initiation complexes leading to global mRNA translational attenuation ⁽⁹⁵⁾. Thereby decreasing protein influx to the ER in support of resolving the cytotoxic threat from accumulated misfolded proteins ⁽⁹⁶⁾. On the other hand, some mRNAs require eIF2 α phosphorylation for translation such as the mRNA encoding activating transcription factor 4 (ATF4). ATF4 is a b-ZIP transcription factor that regulates several UPR target genes including those involved in ER stress mediated apoptosis such as C/EBP homologous protein (CHOP) ⁽⁹⁵⁾.

Activating transcription factor 6 α (ATF6 α): is a 90 kDa type II transmembrane transcription factor. Upon ER stress conditions, ATF6 α transits to the Golgi where it is cleaved by site 1 (S1) and site 2 (S2) proteases, resulting in the release of the cytosolic bZIP transcription factor domain from the Golgi membrane. This processed form of ATF6 α translocates to the nucleus to activate UPR genes involved in protein folding, processing, and degradation (Fig 6) ⁽⁹⁵⁾.

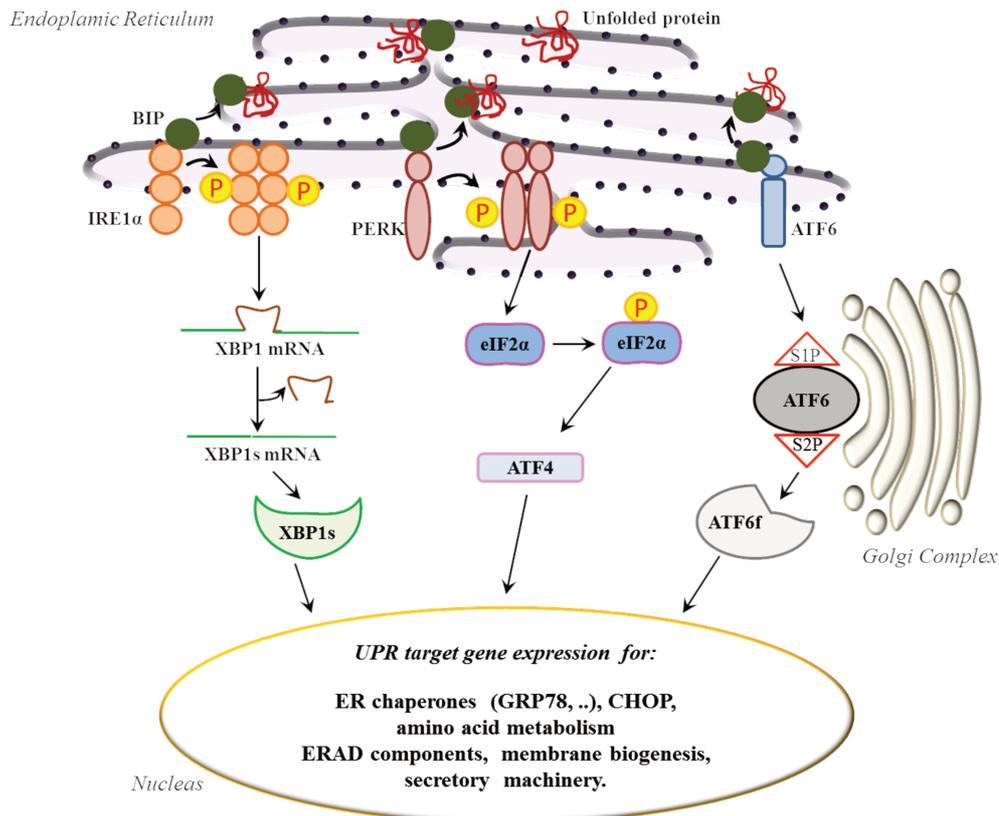


Figure 6. Overview of the three signaling branches of the ER stress response, the UPR. (Referring to Osowski et al, 2010 ⁽⁹⁵⁾ & Harding et al, 2000 ⁽⁹⁶⁾). In the absence of ER stress, ER luminal GRP78 associates with ER transmembrane proteins IRE1, PERK, and ATF6 α to block their activation. Upon ER stress, accumulating unfolded and misfolded proteins inside the ER sequester GRP78, thus dissociating this master regulator from all three transmembrane sensors and relieving their blockage. Activation of IRE1 requires its homodimerization and autophosphorylation. Endonuclease activity of activated IRE1 removes an intron from XBP1 mRNA to generate a shorter splice variant that encodes transcription factor XBP1s. Activation of PERK also entails homodimerization and autophosphorylation, leading to phosphorylation of eIF2 α , which in turn activates the protein translation of ATF4. ATF6 α translocates to the Golgi, where it is proteolytically cleaved by S1 and S2 proteases to generate the transcriptionally active ATF6 fragment. All three transcription factors, ATF4, XBP1s, and ATF6f translocate into the nucleus where they regulate the expression of a variety of gene products collectively involved in managing ER stress.

c) Endoplasmic reticulum stress: its role in diseases and a novel target for therapy

A large number of human diseases has been associated with ER protein-folding defects and ER stress/UPR (see Table 3) and therefore is being recognized as an emerging target for therapy. For that different therapeutic purposes including chemical compounds have been proposed in order to alleviate ER stress (see Table 4)⁽⁹⁷⁾

Table 3. Human diseases linked to ER stress

<i>Disease</i>	<i>Linkage to ER stress</i>
Cancer	<ul style="list-style-type: none"> • Tumor-specific microenvironment activates ER stress • Cancer cells display chronic display of ER stress markers • Knockdown of GRP78 or of CHOP affects chemosensitivity
Parkinson's disease	<ul style="list-style-type: none"> • Parkin expression impacts ER stress • ATF4 leads to increase in parkin expression
Atherosclerosis	<ul style="list-style-type: none"> • Oxidized lipids induce ER stress • Hyperhomocysteinemia induces ER stress • Cholesterol loading induces ER stress-mediated cell death • Reduced plaque necrosis in mice lacking CHOP
Heart disease	<ul style="list-style-type: none"> • ER stress contributes to cardiomyocyte apoptosis • Activation of ER stress in infarcted mouse heart • GRP78 and GRP94 protect against ischemic injury
HBV and HCV infection	<ul style="list-style-type: none"> • HBV induces GRP78 and GRP94 • HCV suppresses IRE1/XBP1 pathway
Type 2 Diabetes	<ul style="list-style-type: none"> • Obesity induces ER stress • Hyperlipidemia and hyperglycemia induce ER stress • Free fatty acids (palmitate) induce beta cell apoptosis • Deletion of CHOP improves beta cell function and survival
Alcoholic liver disease	<ul style="list-style-type: none"> • Alcohol induces ER stress

Table 4. Compounds with potency to ameliorate ER stress.

<i>Compound</i>	<i>Target/effect</i>
Chemical chaperones (TUDCA, 4-PBA)	<ul style="list-style-type: none"> • Increased protein folding capacity • Increased ERAD efficiency • Reduced caspase activation
Inducers of chaperone activity (lithium, valproate, BIX)	<ul style="list-style-type: none"> • Increased expression and activity of chaperones • Supportive of cell-protective ER stress mechanism
Benzodiazepines	<ul style="list-style-type: none"> • Inhibition of ASK1 function and IRE1-ASK1 signaling • Obstructive to pro-apoptotic ER stress mechanism
Inhibitors of eIF2-alpha phosphatase (salubrinal, guanabenz)	<ul style="list-style-type: none"> • Inhibition of PP1/GADD34 phosphatase activity • Supportive of attenuated global protein synthesis
Antioxidants (BHA, TM2002, and baicalein)	<ul style="list-style-type: none"> • Sequestration of free radicals • Reduction of oxidative stress and apoptosis
Inducers of antioxidant pathways (carnosic acid, triterpenoids)	<ul style="list-style-type: none"> • Stimulation of NRF2 pathway • Protection from oxidative stress and resulting apoptosis
Stress kinase inhibitors (JNK or p38 inhibitors)	<ul style="list-style-type: none"> • Inhibition of pro-apoptotic JNK or p38 pathways • Antagonism to pro-apoptotic CHOP function

d) ER stress is involved in MetS and endothelial dysfunction

Studies on obese rodents show that UPR markers, such as PERK, eIF2 α , and BIP are overexpressed in their livers, and adipose tissues, compared to control ones⁽⁹⁸⁾. Investigations made on obese persons presented the activation of ATF6, eIF2 α , IRE1 and XBP1, markers of stress⁽⁹⁹⁾. Also, the induction of the ER stress and the UPR is associated with the dysregulation of lipid and lipoprotein metabolism in MetS⁽¹⁰⁰⁾. These data reveals the implication of ER stress in MetS through different mechanisms. For example, increased levels of cholesterol and saturated fatty acids present in obese individuals decrease the fluidity of ER membrane, leading to the inhibition of the SERCA Ca²⁺-ATPases, depletion of ER luminal Ca²⁺ stores, inhibition of ER-resident molecular chaperones, and the accumulation of unfolded proteins in the ER⁽¹⁰¹⁾. From the other side, experiments show the involvement of ER stress in endothelial dysfunction. Endothelial cells from athero-susceptible regions in normal swine aorta exhibited elevated UPR components, ATF6, IRE1, and XBP1⁽¹⁰²⁾. This effect entangles different mechanisms. Kassan et al⁽⁷³⁾ showed an improvement in the endothelium-dependent relaxation in the aortas of angiotensin II- induced hypertensive mice upon their treatment with tauroursodeoxycholic acid (Tudca) or 4-phenylbutyrate (4-PBA), ER stress inhibitors (mentioned in Table 4), increased eNOS phosphorylation demonstrating that ER stress is an important factor in vascular endothelial dysfunction in hypertension. Another study targeting ER stress-induced endothelial dysfunction in bovine aortic endothelial cells, showed that oxidized and glycated LDL induce oxidative stress in endothelial cells, which in turn induces the UPR through a mechanism involving disturbed ER Ca²⁺ metabolism, resulting in endothelial dysfunction⁽¹⁰³⁾. Galán et al⁽¹⁰⁴⁾ showed that ER stress activation by tunicamycin, led to vascular endothelial dysfunction through p38 MAPK dependent mechanism, reduced ERK1/2 phosphorylation and oxidative stress in endothelial cells from coronary arteries.

e) ER stress partners in crime: mitochondria and oxidative stress

1. Mitochondria histology and function

Mitochondria are double-membraned intracellular organelles that are responsible of energy production for vital metabolic reactions and cellular homeostasis⁽¹⁰⁵⁾. Mitochondria are also implicated in main cellular functions such as Ca²⁺ homeostasis, heme-biosynthesis, steroid hormone biosynthesis, nutrient metabolism, ammonia removal, and activation of signaling pathways⁽¹⁰⁶⁾ involved in innate immunity, autophagy and cell death⁽¹⁰⁷⁾, ROS and NO

production⁽¹⁰⁸⁾. Indispensable for this crucial role of mitochondria is the presence of the mitochondrial respiratory chain and the oxidative phosphorylation (OXPHOS) that transduce electron transport into energy generation in the form of ATP upon ADP phosphorylation⁽¹⁰⁵⁾. However, the primary factor that initiates the dysfunction of mitochondria has been proposed to be the defects in OXPHOS which can further enhance the production of ROS⁽¹⁰⁹⁾.

2. Mitochondria and mitochondrial reactive oxygen species as mediators of metabolic alterations and endothelial dysfunction

Endothelial cells have low mitochondria content compared to cardiomyocytes and hepatocytes; however, mitochondrial dynamics act as a pivotal orchestrator of endothelial cell homeostasis under normal conditions. Damage of mitochondrial dynamics and biogenesis participates in endothelial dysfunction and diverse vascular diseases⁽¹¹⁰⁾. In endothelial cells, where anaerobic glycolysis covers the majority of the cytosolic energy demand, mitochondrial functions are shifted towards signaling phenomena rather than ATP production⁽¹¹¹⁾. Similarly, the intracellular distribution of mitochondria differs depending on the type of endothelial cell and reflects their important signaling role in the regulation of cell-specific processes such as ROS-dependent gene expression or modulation of local Ca²⁺ concentrations and signaling⁽¹¹²⁾, and mostly its communication with other cellular organelle, especially the ER⁽¹¹³⁾.

Furthermore, mitochondrial morphology is dynamic and sensitive to metabolic alterations⁽¹¹⁴⁾. Metabolic status can dramatically affect the form and function of mitochondria, which consequently influences the organ function⁽¹¹⁵⁾. Mitochondrial fragmentation and the subsequent loss of mitochondrial networks have been described in endothelial cells from diabetic patients as well as in cultured human aortic endothelial cells exposed to high glucose concentration⁽¹¹⁶⁾. Altered mitochondrial membrane potential is an important factor that triggers excess mitochondrial ROS (mROS) production in the setting of risk factors, including aging, hypercholesterolemia, hyperglycemia, smoking, and hypoxia. Different sources of mROS have been identified in endothelial cells one such example are complexes I and III of the mitochondrial respiratory chain⁽¹¹⁷⁾.

Clinical investigations suggest that many vascular diseases are accompanied with elevated mROS levels. One typical mechanism by which mROS participates in endothelial dysfunction and subsequent vascular diseases is by uncoupling the mitochondrial eNOS (Fig 7). As a consequence of eNOS uncoupling, NO production is reduced and the pre-existing oxidative stress is enhanced, which contribute significantly to endothelial dysfunction and vascular diseases⁽¹¹⁸⁾.

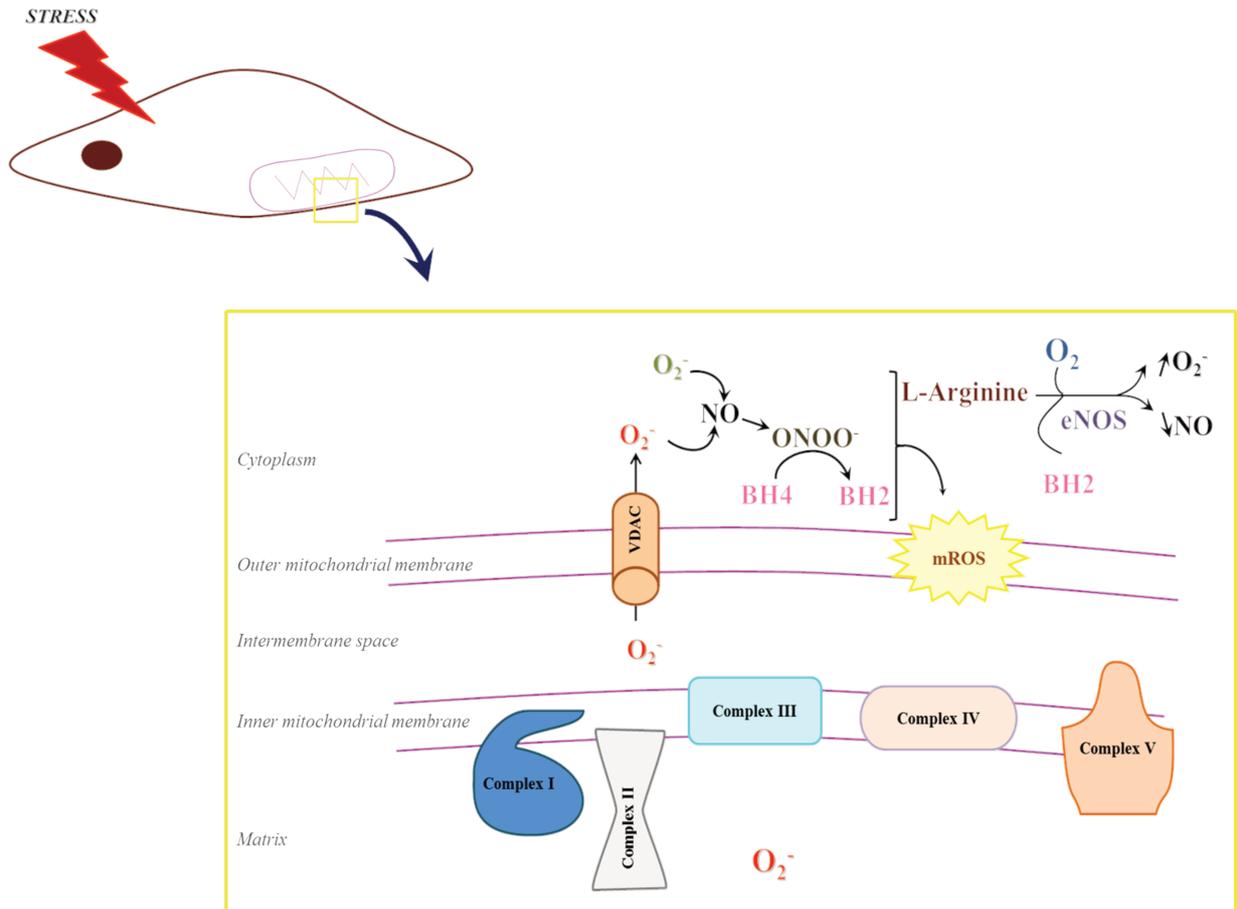


Figure 7. Uncoupling the endothelial NO synthase (eNOS); the mechanism through which ROS participates in endothelial dysfunction and subsequent vascular diseases. (Adapted from Li et al, 2013 ⁽¹¹⁸⁾). Under stress conditions, O_2^- generation is increased. O_2^- reacts with NO to form $ONOO^-$ which together with ROS production leads to mitochondrial dysfunction as evidenced by increased mROS production. Tetrahydrobiopterin (BH₄), a cofactor of eNOS, can be oxidized by $ONOO^-$ resulting in eNOS uncoupling. As a consequence, NO production is reduced and the pre-existing oxidative stress is enhanced, which contribute significantly to endothelial dysfunction.

3. ER and mitochondrial interaction

Arising data highlight the major role of mitochondrial dynamics with ER, and mitochondria/ER interaction in signaling pathways from one hand and in their homeostasis maintenance from the other hand ⁽¹¹⁹⁾.

It is well established that ER and mitochondria collaborate between them through the physiological contact sites contributing for the exchange of metabolites such as lipids and Ca^{2+} between both organelles ⁽¹²⁰⁾. These interconnections called mitochondria-associated membranes (MAMs) participate in the dialogue between ER and mitochondria ⁽¹²¹⁾ and play a major role in tethering and saving homeostasis and signaling pathways between the two organelles ⁽¹²⁰⁾. Out of these interconnections, there is the mitochondrial voltage-dependent anion channel (VDAC) which interacts with the ER-resident inositol triphosphate receptor (IP3R) through the cytosolic chaperone glucose regulated protein 75 (GRP75) ⁽¹²²⁾, in order to allow the exchange of Ca^{2+} through a mitochondrial Ca^{2+} uniport (MCU) ⁽¹²³⁾, and the homotypic and heterotypic interactions of ER-bound mitofusin-2 (Mfn2) with mitochondrial Mfn2 and mitofusin-1 (Mfn1) ⁽¹²⁴⁾ (Fig 8). Mfn2 or Grp75 knock down leads to a decrease in ER-mitochondria Ca^{2+} exchange, proposing that the connection between the two organelles is reduced ⁽¹²²⁾.

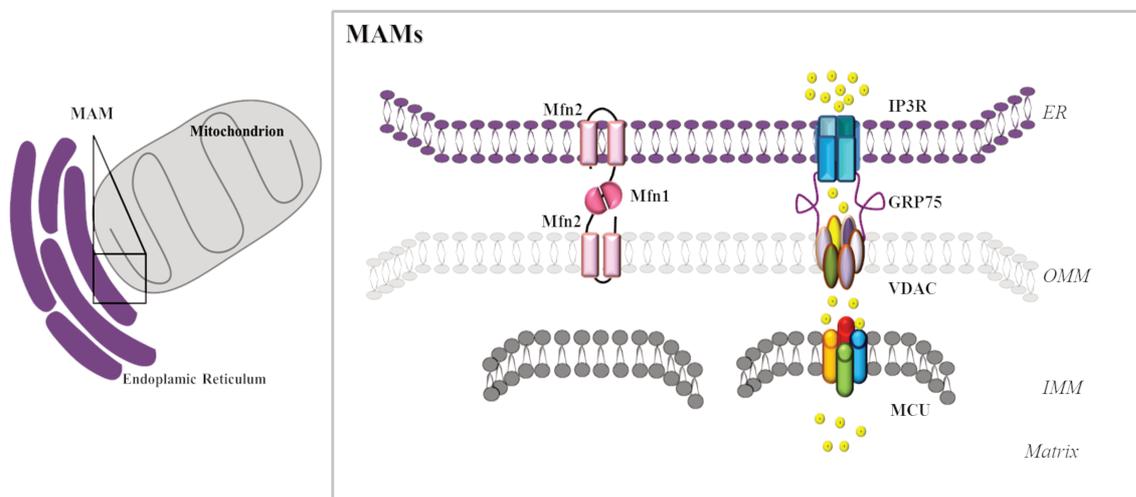


Figure 8. Mitochondria-associated endoplasmic reticulum complex.

(Modified from Rizzuto et al, 2012 ⁽¹²⁵⁾). Mitofusin 2 (Mfn2) is involved in ER-mitochondria tethering, by both homotypic interactions and heterotypic interactions with Mfn1. The voltage-dependent anion channels (VDAC), located at the outer mitochondrial membrane (OMM), is responsible for the rapid transfer of Ca^{2+} from the ER-inositol-1, 4, 5- trisphosphate (IP3R) through the GRP75 (75 kDa glucose-regulated protein), thus facilitating mitochondrial Ca^{2+} uptake, and their function results in high Ca^{2+} in the mitochondria intermembrane space (IMM). Accumulation of Ca^{2+} into the mitochondrial matrix occurs via the mitochondrial Ca^{2+} uniporter (MCU).

Furthermore, recent data showed that during ER stress, the PERK signaling pathway regulates mitochondrial function ⁽¹²⁶⁾. PERK has been proposed to interact with mitochondrial Mfn2, indicating its role in stabilizing ER-mitochondrial contacts ⁽¹²⁷⁾. Consistent with this, genetic depletion of PERK disturbs ER morphology and reduces the number of ER-mitochondrial contacts ⁽¹²⁸⁾. PERK deficient cells show defects in regulating electron transport chain activity reflected by increased basal and maximal respiration ⁽¹²⁷⁾. Moreover, PERK signaling regulates mitochondrial proteostasis and function during ER stress that can lead to mitochondrial dysfunction and promote cellular pathology. In addition, rapid induction of ER stress response with tunicamycin induces changes in MAM indicating that ER stress regulates the dialogue with mitochondria ⁽¹²⁶⁾. In fact, mitochondrial dysfunction has been directly linked to the ER stress response in liver ⁽¹²⁹⁾. Finally, Lu et al⁽¹³⁰⁾ have found that high-fat diet rich in saturated fatty acid palmitate induces swollen mitochondria and extends ER in endothelial cells of mouse aorta.

4. ER and oxidative stress dialogue

Increased ROS production is associated with the overexpression of ER stress markers, the perturbations in the ER-mitochondria cross talk and endothelial dysfunction. This was demonstrated by He X et al ⁽¹³¹⁾ in endothelial cells exposed to hypoxia/ reoxygenation injury. Moreover, in the case of hypertension, ER stress induction along with increased oxidative stress and decreased antioxidant activity, were beyond endothelial dysfunction occurrence in methionine-enriched rats ⁽¹³²⁾. Moreover, in hypertension, ER stress was found to induce endothelial dysfunction through increasing NADPH oxidase activity ⁽¹⁰⁴⁾. On the other hand, Li and colleagues ⁽¹³³⁾ found that high concentrations of uric acid, being a major determinant of total antioxidant capacity of plasma, induced intracellular ROS accumulation, likely triggering ER stress, all of which led to decreased eNOS activity and endothelial dysfunction induction.

These data illustrate the collaborating role of ER, mitochondria and oxidative stress in inducing endothelial dysfunction in metabolic disorders.

AIM OF THE STUDY

In this work, we aimed to study the mechanism through which the two types of MPs, MPs obtained by *in vitro* apoptotic treatment of T lymphocytes and MPs from MetS patients, induce endothelial dysfunction. Referring to the literature, we proposed the ER stress as a possible candidate associated with such model. For this, we tracked the UPR pathways and studied the evolution of its components upon the treatment of human aortic endothelial cells with MPs in the presence or absence of the ER stress inhibitor Tudca. Furthermore, we issued to evaluate the implication of oxidative stress in our studies. For that, we checked the production of cytosolic and mitochondrial ROS at different times. Moreover, several inhibitors of ROS sources (inhibitors of NADPH oxidase, xanthine oxidase, mitochondrial complex I inhibitor, and eNOS inhibitor) and the mitochondrial ROS scavenger, were used to identify ROS origin. Furthermore, we attempted to see the possible perturbations occurring in the mitochondria and its possible participation in decreasing NO bioavailability and therefore inducing endothelial dysfunction. Besides, we tested the expression of the two ingredients Mfn2 and VDAC1 of the ER and mitochondrial interactions, the MAMs. In addition, we pursued to figure out the probable interactions occurring between MPs and endothelial cells. Thus, we investigated the engagement of Fas/FasL and LDL receptor in our model by neutralizing the Fas L present on MPs from one side and LDL receptor found on endothelial cells. To finalize the pathway, we tried to realize the influenced piece of Fas and LDL receptors activation. For that purpose, we examined neutral SMase implication through targeting neutral SMase expression by siRNA and checking its consequence effect.

ARTICLE

ORIGINAL RESEARCH COMMUNICATION

Temporal cross-talk between endoplasmic reticulum and mitochondria regulates oxidative stress and mediates microparticle-induced endothelial dysfunction

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Abstract

Aims: Circulating microparticles (MPs) from metabolic syndrome patients and those generated from apoptotic T-cells induce endothelial dysfunction; however, the molecular and cellular mechanism(s) underlying in the effects of MPs remain to be elucidated. *Results:* Here, we show that both types of MPs increased expression of endoplasmic reticulum (ER) stress markers XBP-1, p-eIF2alpha and CHOP and nuclear translocation of ATF6 on human aortic endothelial cells. MPs decreased *in vitro* nitric oxide release by human aortic endothelial cells, whereas *in vivo* MP injection into mice impaired the endothelium-dependent relaxation induced by acetylcholine. These effects were prevented when ER stress was inhibited suggesting that ER stress is implicated in the endothelial effects induced by MPs. MPs affected mitochondrial function and evoked sequential increase of cytosolic and mitochondrial reactive oxygen species (ROS). Pharmacological inhibition of ER stress and silencing of neutral sphingomyelinase with siRNA abrogated all MP-mediated effects. Neutralization of Fas-Ligand carried by MPs abolished effects induced by both MP types, whereas neutralization of low density lipoprotein-receptor on endothelial cells prevented T-lymphocyte MP-mediated effects. *Innovation and Conclusion:* Collectively, endothelial dysfunction triggered by MPs involves temporal cross-talk between ER and mitochondria with respect to spatial regulation of ROS via the neutral sphingomyelinase and interaction of MPs with Fas and/or low density lipoprotein-receptor. These results provide a novel molecular insight into the manner MPs mediate vascular dysfunction and allow identification of potential therapeutic targets to treat vascular complications associated with metabolic syndrome.

Introduction

Metabolic syndrome defines a cluster of interrelated risk factors for cardiovascular disease and diabetes. These factors include metabolic abnormalities such as hyperglycemia, elevated triglyceride levels, low high-density lipoprotein cholesterol levels, high blood pressure and obesity, mainly central adiposity. Endothelial dysfunction participates actively in the development of cardiovascular diseases associated with metabolic syndrome (17). Among biological markers of endothelium injury, microparticles (MPs) have been involved in the pathogenesis and maintenance of cardiovascular, metabolic, and inflammatory diseases (9). Indeed, these small vesicles, released from plasma membrane of activated or apoptotic cells, carry proteins, nucleic acids and lipids that can modify phenotype and function when delivered to target cells (21). In particular, we have previously described that MPs from both apoptotic T cells and from metabolic syndrome patients induced endothelial dysfunction characterized by a decrease of nitric oxide (NO) release associated with the inhibition of endothelial NO-synthase (eNOS), and an increase in oxidative and nitrative stresses in human endothelial cells (2, 25). In addition, when MPs from either apoptotic T cells or metabolic syndrome patients were injected into mice, an impairment of endothelium-dependent vasorelaxation in response to acetylcholine was observed illustrating their pathophysiological relevance (2, 25). However, the molecular and cellular mechanism(s) underlying in the effects of MPs remain to be elucidated.

Several reports have shown that the activation of endoplasmic reticulum (ER) stress response plays an important role in the pathogenesis of metabolic disorders such as insulin resistance, type 2 diabetes and obesity in animals (3, 26) as well as in humans (5). ER stress is induced by the accumulation of misfolded proteins in the ER lumen which leads to the activation of unfolded protein response (UPR). UPR is initiated by three main ER transmembrane stress sensors: pancreatic endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 alpha

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3 (IRE1 α), and activating transcription factor 6 (ATF6) (7). The UPR is initially activated as a
4 pro-survival mechanism, however, prolonged UPR activation in conditions such as obesity
5 and metabolic syndrome leads to apoptosis, oxidative stress, and inflammation and is referred
6 to as the “ER stress response”. Recently, Galán et al (10) have demonstrated that chemical
7 induction of ER stress with tunicamycin is associated with endothelial dysfunction and
8 increased oxidative stress in the arterial wall. Under pathophysiological conditions such as
9 hypertension, ER stress was reported to be involved in endothelial dysfunction induced by
10 angiotensin II infusion (12). Furthermore, several lines of evidence strongly support the link
11 between mitochondrial dysfunction and cardiovascular complications associated with
12 metabolic syndrome. In the skeletal muscle of type 2 diabetic patients, a downregulation of
13 PGC1 α -responsive genes involved in oxidative phosphorylation has been described (24).
14 Also, both basal and maximal ADP-stimulated respirations were decreased in type 2 patients
15 (22, 28). Similar results have been observed in human cardiac muscle (23). Moreover, an
16 important increase in ER-mitochondria contact sites via the mitochondria-associated
17 membranes (MAMs) have been described during high-fat diet-induced obesity leading to
18 significant increase in hepatic lipid accumulation and decreased mitochondrial oxidative
19 function (3). In parallel, elevated reactive oxygen species (ROS) production in cytosol,
20 through the overexpression of the NADPH isoform NOX4, and in mitochondria have been
21 detected in smooth muscle aortic cells from atherosclerotic aged donors leading to impaired
22 mitochondrial function (32).

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The aim of the present study is to decipher how MPs transfer their message to induce
endothelial dysfunction. Detailed analyses of the mechanisms involved with special interest in
the cross-talk between ER and mitochondria in the regulation of oxidative stress were
conducted. This would help underpinning new molecular and cellular pathways governing the
vascular effects of MPs and will pave the way for identifying novel potential targets against

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MP-induced endothelial dysfunction, which predisposes to the initial pro-atherosclerotic events transforming metabolic syndrome to different vascular diseases.

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Results

T lymphocytic MPs induce endothelial dysfunction through ER stress response activation

In response to treatment by MPs from apoptotic lymphocytes, UPR pathway activation was assessed using the ER stress inhibitor tauroursodeoxycholic acid (TUDCA), a chemical chaperone known to enhance adaptive response of ER. MPs activated the three canonical pathways associated with ER stress response: PERK, IRE1 α and ATF6. Indeed, MPs resulted in an increased phosphorylation of PERK and eIF2 α , as well as mRNA (not shown) and protein CHOP expressions (Fig. 1A-C). In addition, MP treatment enhanced X-box binding protein 1 (XBP1) splicing downstream to IRE1 α (Fig. 1D) and induced the nuclear translocation of ATF6 (Fig. 1E). Importantly, all these MP-evoked effects were prevented in the presence of the ER stress inhibitor TUDCA (Fig 1A-E). It should be noted that the ER stress inducer tunicamycin was able to activate the same three classical ER stress pathways, which were partially prevented by TUDCA (Fig. S1). Altogether, these data indicate that MPs from apoptotic lymphocytes lead to the activation of all three arms of ER stress response.

In order to verify whether ER stress response activation accounts for the endothelial dysfunction induced by T lymphocyte MPs (25), *in vitro* NO $^{\cdot-}$ production was evaluated in the absence or the presence of TUDCA. As previously described, T lymphocyte MPs significantly decreased NO $^{\cdot-}$ production in human aortic endothelial cells (HAoECs) (Fig. 1F). Interestingly, the inhibition of ER stress with TUDCA abolished the ability of MPs to reduce NO $^{\cdot-}$ production. In order to establish the pathophysiological relevance of MPs, endothelium-dependent relaxation in response to acetylcholine was evaluated following MP incubation with mouse aortic rings in the absence or in the presence of TUDCA. MPs significantly reduced the maximal acetylcholine-evoked relaxation, which was improved by TUDCA (Fig. 1G). Finally, similarly to MPs, tunicamycin was able to reduce both *in vitro* NO $^{\cdot-}$ release by

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3 endothelial cells and acetylcholine-induced relaxation of mouse aortic rings. These effects
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5 were partially prevented in the presence of TUDCA (Fig. S1).
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7 Collectively, these results indicate that the activation of ER stress response is implicated in
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9 endothelial dysfunction induced by T lymphocyte MPs.
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11 *Involvement of membrane receptors in T lymphocyte MP-induced ER stress in endothelial*
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13 *cells*
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16 We have previously shown that T lymphocyte MPs, through their Fas-Ligand (FasL), induce
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18 vascular smooth muscle cell inflammation by directly interacting with Fas of the recipient
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20 cells (31). Also, Yang et al (33) have reported the involvement of low density lipoprotein
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22 receptor (LDL-R) in the effects induced by T lymphocyte MPs on human retinal endothelial
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24 cells. In order to determine the mechanism(s) by which MPs would induce ER stress in
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26 HAoECs, the implication of both Fas/FasL interaction and LDL-R was analyzed on MP-
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28 induced eIF2 α phosphorylation. Interestingly, we observed that neutralizing either FasL on
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30 MPs or LDL-R on endothelial cells significantly reduced the MP-induced phospho-eIF2 α
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32 (Fig. 2A). Altogether, these data indicate that both Fas/FasL interaction and LDL-R pathway
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34 are involved in MP-mediated ER stress induction.
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38 *Neutral sphingomyelinase (SMase) is the link between MP-activated receptor and ER stress*
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40 *response induction*
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43 Since SMase cascade has been implicated in the regulation of ER stress response (15), we
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45 assessed the involvement of SMase on MP-induced effects. MP treatment induced an increase
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47 of neutral SMase expression, which was prevented after neutralizing FasL on MPs (Fig. 2B)
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49 or when LDL-R on endothelial cells was blocked (Fig. 2C). Treatment of endothelial cells by
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51 MPs for 24 hours, but not for 4 hours, increased phospho-eIF2 α . Interestingly, silencing
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53 neutral SMase with a specific siRNA abolished MP-induced increase of eIF2 α
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55 phosphorylation after 24 hours of MP treatment (Fig. 2D and 2E).
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3 Moreover, using either the specific neutral SMase inhibitor GW4869 or siRNA against
4 neutral SMase completely prevented the MP-induced reduction of eNOS activity as reflected
5 by phospho-Ser eNOS/phospho-Thr eNOS ratio (Fig. 2F and 2G). Furthermore, MP-induced
6 NO[•] reduction was abolished when neutral SMase was inhibited with GW4869 or silenced by
7 siRNA (Fig 2H). Altogether, these results suggest that neutral SMase pathway controls the
8 effects of MPs via Fas/FasL and LDL-R interaction upstream of ER stress, eNOS activation
9 and NO[•] production.

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19 *Cytosolic ROS production is upstream of T lymphocyte MP-induced ER stress in endothelial*
20 *cells*

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22 The possible involvement of oxidative stress in T lymphocyte MP-induced ER stress
23 activation was then assessed by incubating HAoECs with various inhibitors the sources of
24 ROS prior to MP stimulation. Indeed, intracellular enzymatic systems with the capacity to
25 generate ROS were inhibited by allopurinol, apocynin, rotenone, or N ω -nitro-L-arginine (L-
26 NA) which are inhibitors of xanthine oxidase, NADPH oxidase, respiratory chain complex I,
27 and NOS, respectively. None of the ROS inhibitors modified the phosphorylation of eIF2 α at
28 basal levels (Fig. 3A) whereas anyone significantly decreased eIF2 α phosphorylation at 24
29 hours of MP treatment. These results suggest that MP-induced ROS production from different
30 sources is required for the activation of ER stress response (Fig. 3B).

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42 In order to establish the implication of cytoplasmic and mitochondrial ROS in the effects of
43 MPs, time course (i.e., 2, 4, and 24 hours) changes of ROS levels were measured. Analysis of
44 ROS levels, using electronic paramagnetic resonance (EPR) and dihydroetidium (DHE)
45 labeling, showed that T lymphocyte MPs had no effect on ROS production at 2 hours,
46 increased at 4 hours and returned to basal levels at 24 hours (Fig 3 C-I). This effect of T
47 lymphocyte MPs on ROS production was not affected by TUDCA. It is important to note that
48 tunicamycin treatment induced similar patterns of ROS production as those obtained with
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3 MPs (Fig. S2). Interestingly, except for the mitochondrial ROS scavenger (mito-TEMPO),
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5 none of the ROS inhibitors tested (allopurinol, apocynin, rotenone and L-NA), reduced the
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7 early increase of cytosolic ROS production (Fig. 3J). To further analyze the enzyme involved
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9 in this ROS production, we found that lymphocyte MPs increased the expression of p47phox
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11 expression, a regulatory subunit of NADPH oxidase at 4 hours, but not at 24 hours of
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13 treatment (Fig. 3 K). This increase was completely prevented after silencing of neutral SMase
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15 (Fig. 3 K).
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18 Altogether, lymphocyte MPs activate neutral SMase and induce an early increase of cytosolic
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20 ROS production, upstream of ER stress activation via at least the NADPH oxidase activation.
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23 *Cross-talk between ER and mitochondria is involved in the effects induced by lymphocyte*
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25 *MPs in endothelial cells*
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27 To monitor mitochondrial ROS production, MitoSox probe was used. Quantification of
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29 confocal images showed that mitochondrial ROS fluorescence was reduced at 2 and 4 hours,
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31 but was greatly enhanced after 24 hours of lymphocyte MP treatment (Fig. 4A-D).
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33 Interestingly, the ER stress inhibitor TUDCA did not modify the reduction of MitoSox
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35 fluorescence at 2 and 4 hours but completely abolished the increase at 24 hours. These results
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37 suggest that lymphocyte MPs induce a late increase of mitochondrial ROS by a mechanism
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39 downstream of ER stress activation. In order to confirm this, the mitochondrial ROS
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41 scavenger mito-Tempo was used and did not show any effect on the increase of phospho-
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43 eIF2 α induced by lymphocyte MPs (Fig. 4E).
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46 The effects of MPs on mitochondrial O₂ consumption were then analyzed in order to decipher
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48 the role of mitochondria on the MP-induced endothelial dysfunction. Basal O₂ consumption
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50 was not modified either by MPs or MPs + TUDCA (Fig. 4F). However, MP treatment
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52 significantly reduced both oligomycin and FCCP mitochondrial respirations which were
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54 abolished when ER stress was inhibited with TUDCA (Fig. 4G and 4H). These findings
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3 suggest that MPs are able to modify mitochondrial respiration by a mechanism implicating
4 ER stress. Exposure to MPs resulted in an increase of complex IV activity (Fig. 4I) but had no
5 effect on the activity of the other complexes of the electron transport chain (not shown). To
6 confirm the interaction between ER and mitochondria through the formation of tight physical
7 junctions, MAM integrity was analyzed following MP treatment. As shown in Figure 4J and
8 4K, MPs induced MAM disruption characterized by an overexpression of mitofusin 2 (Mfn2)
9 and a decreased expression of voltage-dependent anion channel 1 (VDAC1). Moreover, these
10 effects were prevented in the presence of the ER stress inhibitor TUDCA suggesting that MP-
11 induced ER stress leads to MAM disruption on endothelial cells. Finally, to verify whether the
12 MP effects on mitochondria lead to endothelial dysfunction, NO[•] production was measured in
13 the presence of mito-Tempo. Scavenging mitochondrial ROS with mito-Tempo abolished the
14 decrease in NO[•] release induced by MPs (Fig. 4L). This result suggests that alteration of
15 mitochondrial function with respect to respiration and ROS production, as a consequence of
16 ER stress, leads to endothelial dysfunction.

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34 *MPs from metabolic syndrome patients induce endothelial dysfunction via Fas/FasL pathway*
35 *and neutral SMase activation leading to ER stress activation in vitro and ex vivo*

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38 We have previously described that MPs from metabolic syndrome patients, but not those of
39 healthy subjects, induced endothelial dysfunction (2). Here, we tested whether molecular
40 pathways involved in the effects of MPs from metabolic syndrome required also the cross-talk
41 between ER and mitochondria and oxidative stress similarly to lymphocyte MPs. Therefore,
42 MPs from metabolic syndrome patients or healthy subjects were isolated for whom detailed
43 clinical information is described in Supplementary Table 1. As expected, metabolic syndrome
44 patients showed greater visceral obesity as given by waist circumference, enhanced
45 triglyceridemia, and increased blood pressure.

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3 The total number of circulating MPs was significantly increased in patients with metabolic
4 syndrome compared to healthy subjects (Supplementary Table 2). Phenotypical
5 characterization of cellular origin of MPs showed an increase of ~6-fold (annexin V⁺), ~3.3-
6 fold (CD41⁺), and ~9.6-fold (CD146⁺) of the circulating level of procoagulant-, platelet-, and
7 endothelial-derived MPs in metabolic syndrome patients compared to healthy subjects,
8 respectively. Also, levels of erythrocyte- and leukocyte-derived MPs (CD235a⁺, CD45⁺) were
9 elevated (~9 and ~2.7-fold, respectively) in these patients.
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12 As shown in Fig. 5A and 5B, MPs from metabolic syndrome patients, but not from healthy
13 individuals, induced an increase in phosphorylation of eIF2 α and nuclear translocation of
14 ATF6, which were prevented in the presence of TUDCA. Similarly and as expected, MPs
15 from metabolic patients, in comparison to healthy subjects, caused a reduction in NO[•] release
16 from endothelial cells, which was abolished when ER stress was inhibited by TUDCA (Fig.
17 5C). Moreover, incubation of mouse aortic rings with MPs from metabolic syndrome patients,
18 but not from healthy subjects, was associated to a reduced endothelium-dependent relaxation
19 in response to acetylcholine (Fig. 5D). This effect was prevented when vessels were incubated
20 in the presence of TUDCA. Taken together, these results indicate that ER stress plays a key
21 role in the induction of endothelial dysfunction induced by MPs from metabolic syndrome
22 patients.
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26 In order to establish the receptor involved in the effects of MPs from metabolic syndrome
27 patients on endothelial cells, we neutralized either FasL on MPs or LDL-R on HAoECs.
28 Interestingly, neutralization of FasL but not LDL-R on HAoECs, prevented the
29 phosphorylation of eIF2 α by metabolic syndrome MPs (Fig. 5E) and abolished their ability to
30 enhance the expression of SMase (Fig. 5F). Finally, it is important to note that silencing
31 neutral SMase with siRNA significantly abolished the increase of eIF2 α phosphorylation by
32 metabolic syndrome MPs both at early (4 hours) and late stage (24 hours) of treatment (Fig.
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3 5G and 5H), in contrast to T lymphocyte MPs which were able to increase eIF2 α at 24 hours
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5 only (Fig. 2E).

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7 *Cytosolic and mitochondrial ROS productions are downstream of metabolic syndrome MP-*
8
9 *induced ER stress in endothelial cells*

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11 As illustrated in Figure 6A-H, MPs from metabolic syndrome induced an oxidative stress by
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13 increasing both cytosolic and mitochondrial ROS. DHE staining showed that MPs from
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15 metabolic syndrome patients, in comparison to healthy subjects, enhanced cytosolic ROS at 2
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17 and 4 hours, but not at 24 hours (Fig. 6A-D). Also, MPs from metabolic syndrome patients
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19 but not those from healthy subjects increased MitoSox fluorescence at 24 hours, but not at 2
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21 or 4 hours of treatment of HAoECs (Fig. 6E-H). Cytosolic ROS, but not mitochondrial ROS
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23 production, was abolished in the presence of ER stress inhibitor TUDCA. Interestingly, MPs
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25 induced p47phox expression at 4 hours, but not at 24 hours, which was abolished after SMase
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27 silencing (Fig. 7A and 7B). These results suggest that MPs from metabolic syndrome patients
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29 activate neutral SMase and induce a spatio-temporal increase of cytosolic ROS at early stage
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31 via the NADPH oxidase that concur to ER stress and mitochondrial ROS at late stages of
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33 stimulation. All of these effects participate to metabolic syndrome MP-induced endothelial
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35 dysfunction. All of these effects participate to metabolic syndrome MP-induced endothelial
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Discussion

In the present study, we provide evidence to support the crucial role of ER and mitochondria interaction in the deleterious effects of human MPs on endothelial function. Most in detail, MPs from apoptotic T cells act on Fas and LDL-R, then, via neutral SMase cascade induce cytosolic ROS production which activates ER stress. Through the interaction between ER and mitochondria, mitochondrial ROS are increased and contribute to decreased bioavailability of NO and the subsequent impairment of endothelium-dependent vasorelaxation. Concerning MPs from metabolic syndrome patients, Fas/FasL, but not LDL-R, participates in the response induced by MPs. In addition, neutral SMase activation induces directly ER stress which, in turn, increases both cytosolic and mitochondrial ROS. All of these events lead to reduction of NO release and the subsequent impairment of endothelium-dependent vasorelaxation.

MPs have been described as cargo to delivery biological information between cells. We have previously described that MPs from either apoptotic T cells or metabolic syndrome patients induce endothelial dysfunction through the inhibition of eNOS (2, 25). In the present study, we show that both types of MPs induce endothelial dysfunction by ER stress activation. Thus, in a similar manner to tunicamycin, MPs activate the three canonical UPR pathways as illustrated by the increased phosphorylation of PERK and eIF2alpha, XBP1 splicing and nuclear ATF6 translocation. ER stress activation has been described in endothelial cells of obese patients (11) and after an intralipid infusion in endothelial cells from healthy subjects (30), suggesting that metabolic disorders can lead to ER stress that may be relevant to vascular diseases. In the present study, the involved mechanisms in the ER induction of MPs from T cells and those from metabolic syndrome patients were found to be slightly different, probably due to the differences between MP origins. Indeed, we show that metabolic syndrome patients display elevated levels of platelet- and non-platelet-derived MPs, mainly

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3 from leukocytes, erythrocytes and endothelial cells. In this respect, we have previously shown
4 that the effects of metabolic syndrome MPs on endothelial cells are probably supported by
5 non-platelet-derived MPs since this subset of MPs reduce NO[•] production⁴. In addition,
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7 Chironi et al (6) have found that circulating levels of leukocyte-derived MPs were higher in
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9 metabolic syndrome patients than in healthy individuals and that these levels positively
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11 correlate with the number of metabolic syndrome components.
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16 MPs carry proteins, lipids and nucleic acids, mainly mRNA and miRNA. Since RNase
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18 treatment has no effect in MP-induced responses on HAoECs (not shown), we decided to
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20 analyze the direct interaction between MPs and target cells via the ligand-receptor binding.
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23 We have previously shown that interaction of FasL carried by MPs with Fas at the surface of
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25 target cells mediates the induction of pro-inflammatory proteins in the vascular wall (1, 31),
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27 whereas Yang et al (33) demonstrated the involvement of LDL-R in the MP-induced effects.
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29 Here, we show that both mechanisms participate to the effects of MPs from T cells, but only
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31 Fas/FasL interaction is involved in the effects generated by MPs from metabolic syndrome
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33 patients. Indeed, neutralization of LDL-R on endothelial cells does not affect metabolic
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35 syndrome MP-induced ER stress. Nevertheless, both types of MPs are able to activate neutral
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37 SMase which has been directly related to endothelial dysfunction (for review see 27). In
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39 addition, we provide proof that inhibition of neutral SMase, or silencing its expression,
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41 strongly blocks MP-induced ER stress and improves both eNOS activity and NO[•] production,
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43 illustrating the implication of neutral SMase pathway on MP-induced endothelial dysfunction.
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45 Nonetheless, intracellular pathways involved in the effects of both types of MPs show
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47 differential spatial and temporal regulation with respect to cytosolic and mitochondrial pools
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49 of ROS. Activation of ER stress by MPs from T cells is dependent on ROS production from
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51 different origins since inhibitors of NADPH oxidase, xanthine oxidase, and mitochondrial
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53 complex I prevented phosphorylation of eIF2 α , which suggests a link between the different
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3 ROS sources. Thus, it has been proposed that NADPH oxidase plays a central role in
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5 orchestrating the activation and dysfunction of other enzymes generating ROS (for review see
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7 14). In addition, the early increase (at 4 hours of treatment) on cytosolic ROS production
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9 evoked by MPs from apoptotic T cells was not inhibited by TUDCA, indicating that the
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11 enhancement of cytosolic ROS is upstream to ER stress. Interestingly, silencing SMase
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13 partially prevented the increase of p47phox subunit of NADPH oxidase induced by MPs at 24
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15 hours suggesting that SMase activation precedes NADPH oxidase-dependent deleterious
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17 effects of MPs in endothelial cells. The SMase-p47phox-ROS cascade has been described in
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19 vascular smooth muscle cells under hypoxia conditions (8) and in neurons exposed to TNF-
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21 alpha (4), however, to the best of our knowledge, the relationship of this cascade with ER
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23 stress has never been studied. Furthermore, several studies showed that cellular toxics such as
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25 arsenic or ischemia/reperfusion injury exert their cytotoxicity by inducing apoptosis through a
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27 ROS-induced ER stress which is associated to a mitochondrial dysfunction (29, 34). In the
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29 present study, we provide evidence that mitochondrial ROS increase, changes on
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31 mitochondrial respiration and complex IV activity are detected at late (24 hours) but not early
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33 (4 hours) MP treatment, and are abolished when ER stress is inhibited, indicating that ER
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35 stress activation is mandatory for the generation of mitochondrial ROS and perturbation of
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37 mitochondrial function. Remarkably, scavenging of mitochondrial ROS by mito-Tempo
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39 restores NO[•] production, illustrating the involvement of mitochondrial ROS on the MP-
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41 induced endothelial dysfunction. Moreover, the enhanced MAM alteration, which suggests an
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43 interaction between ER stress and mitochondria, could account for the subsequent
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45 modifications of the mitochondrial function. It is worth noting that ER and mitochondrial
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47 cross-talk has been described in neurodegenerative (13) and metabolic disorders (3, 18)
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49 suggesting that rescuing mitochondrial and ER stress impairments may identify new targets
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51 against these pathologies.
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3 In this respect, activation of SMase pathway by MPs from metabolic syndrome patients
4 directly induces ER stress which on its turn evokes an increase of both cytosolic and
5 mitochondrial ROS. It is important to note that the spatio-temporal regulations of ROS
6 induced by MPs from metabolic syndrome patients are different from those of T lymphocytic
7 MPs. In fact, with respect to the increase of cytosolic ROS, ER stress activation is
8 downstream for the former and upstream for the latter. This differential response probably
9 results from the fact that a mixture of MP subsets triggers the effects of metabolic syndrome
10 MPs although we previously reported that non-platelet MPs are responsible for endothelial
11 dysfunction. Indeed, it is well known that the content of MPs depends on the cells they
12 originate from, the stimulus of production and the mechanism of vesicle generation (9).
13 Nevertheless, the present study emphasizes the importance of ER stress as a key control of
14 oxidative stress leading to endothelial dysfunction by both types of MPs. In this respect, it has
15 been described that the presence of unfolded proteins in the ER lumen is sufficient to activate
16 oxidative stress (for review see 19). This process may be related with the fact that ER stress
17 induction by MPs from metabolic syndrome patients takes place early, only after 4 hours of
18 MP treatment.
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38 Collectively, we demonstrate that endothelial dysfunction triggered by MPs involves temporal
39 cross-talk between ER and mitochondria with respect to spatial regulation of oxidative stress
40 via the SMase route (Figure 8). These events occur via interaction of MPs on endothelial cells
41 with Fas and/or LDL-R. These results highlight novel potential targets to fight against the
42 pivotal role of MPs on endothelial dysfunction leading to the increase of cardiovascular
43 complications including those associated with metabolic syndrome.
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Innovation

In last years, an increasing interest on circulating microparticles, extracellular vesicles released from plasma membrane of cells, as players in the development and maintenance of cardiovascular diseases has been reported. Our study identifies a new pathway involving a spatio-temporal cross-talk between endoplasmic reticulum and mitochondria in the generation of oxidative stress accounting for the impairment of endothelial function induced by microparticles from metabolic syndrome patients and allow identification of potential therapeutic targets to treat vascular complications associated with metabolic diseases.

Materials and Methods

MP production from T-cells. The human lymphoid CEM T-cell line was used for MP production. Cells were seeded at 10^6 cells/ml and cultured in serum-free X-VIVO 15 medium (Lonza, Basel, Switzerland). MPs were produced as previously described (20, 25). Briefly, cells were treated with actinomycin D (1 μ g/ml; Sigma-Aldrich, St Quentin-Fallavier, France) for 24 hours. The supernatant was obtained by centrifugation at 750g for 15 minutes, and then at 1,500g for 5 minutes to remove cells and large debris, respectively. MPs from the supernatant were washed after three centrifugation steps (45 minutes, 14,000g) and recovered in 400 μ l of sterile NaCl 0.9%. Last washing medium was used as vehicle. The determination of the amount of MPs was conducted by measuring MP-associated proteins using the DC Protein Assay (Bio-Rad, Hercules, CA). MPs were used at 10 μ g/ml corresponding to $1,330 \pm 312 \times 10^3$ MPs. This concentration of lymphocyte MPs is found in plasma from patients undergoing carotid endarterectomy ($0-2 \times 10^6$ MPs/ml plasma) (16).

Metabolic syndrome patients. This study was approved by the ethics committee of the University Hospital of Angers (France) (NCT: 00997165). A total of 30 patients were included with metabolic syndrome from the METABOL cohort at the Department of Endocrinology and Nutrition of the University Hospital of Angers. Patients were eligible for inclusion, according to the National Cholesterol Education Program-Adult Treatment Panel III, when they had at least three of the following five criteria : i) waist circumference >102 or 88 cm for men and women, respectively; ii) systolic and diastolic pressures $\geq 130/85$ mmHg; iii) fasting glycemia ≥ 1.1 g/l; iv) triglycerides ≥ 1.5 g/l; and v) high-density cholesterol lipoprotein <0.4 g/l in men or <0.5 g/l in women (Supplementary Table 1). Patients with a history of cardiovascular diseases, preexistent chronic inflammatory disease, and cancer were excluded. Normal controls consisted of 20 subjects who met less than two of the metabolic syndrome criteria (70% without any component of metabolic syndrome).

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3 *MP collection from patients.* Peripheral blood (20 ml) from healthy subjects or patients was
4 collected in EDTA tubes (Vacutainers; Becton Dickinson, Le Pont de Claix, France) from a
5 peripheral vein using a 21-gauge needle to minimize platelet activation and were processed
6 within 2 hours. After a 20-minute centrifugation at 270g, platelet-rich plasma was separated
7 from whole blood which was then centrifuged for 20 minutes at 1500g to obtain platelet-free
8 plasma (PFP). Two hundred μ l of PFP were frozen and stored at -80°C until subsequent use
9 for MP characterization by flow cytometry. Remaining PFP was subjected to three series of
10 centrifugations each at 21,000g for 45 minutes to pellet MPs for *in vitro* studies, and the
11 supernatant was replaced by 200 μ l of sterile 0.9% NaCl and stored at 4°C until subsequent
12 use. MPs from healthy subjects or patients were used at the circulating concentration detected
13 for each individual, as previously described (1, 2).
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27 *Characterization of MP phenotype.* Membrane MP sub-populations were discriminated in
28 PFP according to the expression of membrane-specific antigens (1, 2). Irrelevant human IgG
29 was used as an isotype-matched negative control for each sample. For numeration studies, 10
30 μ l of PFP were incubated with 5 μ l of specific antibody (Beckman Coulter, Villepinte,
31 France). Annexin V binding was used to numerate phosphatidylserine-expressing circulating
32 MPs (2 μ l of annexin V/5 μ l PFP). After 30 minutes at room temperature, samples were
33 diluted in 300 μ l of sterile 0.9% NaCl or annexin-V labeling buffer, respectively. Then, an
34 equal volume of sample and Flowcount beads were added and samples were analyzed in a
35 flow cytometer 500 MPL system (Beckman Coulter).
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Cell culture. Human Aortic Endothelial Cells (HAoECs) were maintained in culture in
endothelial cell growth medium MV2 (Promocell, Heidelberg, Germany) supplemented with
1% streptomycin/penicillin (Lonza). Cells were treated for 24 hours with ER stress inducer
tunicamycin (0.8 $\mu\text{g/ml}$, Sigma-Aldrich), 10 $\mu\text{g/ml}$ of T lymphocyte MPs or those from
healthy subjects or metabolic syndrome patients, in the absence or in the presence of the ER

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3 stress inhibitor, TUDCA (100 $\mu\text{mol/l}$, Sigma-Aldrich). In another set of experiments, 30
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5 minutes before MP treatment, cells were pre-treated with the NADPH oxidase inhibitor,
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7 apocynin (Apo, 100 $\mu\text{mol/l}$, Calbiochem, Darmstadt, Germany), the inhibitor of xanthine
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9 oxidase, allopurinol (Allo, 50 $\mu\text{mol/l}$, Sigma-Aldrich), the inhibitor of mitochondrial complex
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11 I NADH dehydrogenase, rotenone (Rot, 5 $\mu\text{mol/l}$, Sigma-Aldrich), the NO⁻ synthase inhibitor,
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13 L-NA (100 $\mu\text{mol/l}$, Sigma-Aldrich), the mitochondria-targeted scavenger mito-TEMPO (25
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15 nmol/l, Santa Cruz Biotechnology) or the inhibitor of neutral SMase, GW4869 (10 $\mu\text{mol/l}$,
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17 Sigma-Aldrich). All products were used at concentrations at which no cytotoxicity was
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19 observed, as deduced from trypan blue exclusion assay.
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23 *Western Blot.* After treatment, cells were homogenized and lysed. Proteins (40 μg) were
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25 separated on 4-12% NuPage gels (ThermoFisher Scientific, Rockford, IL). Blots were probed
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27 with antibodies against PERK, phospho-PERK, eIF2 α , phospho-eIF2 α , CHOP, phospho-
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29 Thr495 eNOS (Cell Signaling, Danvers, MA), mitofusin 2 (Mfn2), VDAC1 α (Abcam,
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31 Cambridge, United Kingdom), p47-phox and phospho-Ser1177 eNOS (BD Biosciences, San
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33 Jose, CA). A polyclonal mouse anti-human β -actin antibody (Sigma-Aldrich) was used for
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35 standardization of protein gel loading.
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39 In another set of experiments, cells were pre-treated with LDL-R antibody (5 $\mu\text{g/ml}$, R&D
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41 Systems, Minneapolis, MN) or its control IgG antibody for 30 minutes before MP treatment.
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43 Also, FasL carried by MPs was pre- incubated with human anti-FasL (5 μg , BD Biosciences)
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45 for 30 minutes at 4°C to allow neutralization of MP FasL after being washed two times with
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47 NaCl 0.9% in order to remove unbound anti-FasL antibody.
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50 *Nuclear translocation of ATF6 by confocal microscopy.* Cells were seeded on glass slides
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52 (Ibidi, Martinsried, Germany), for 24 hours and then treated with either tunicamycin or MPs
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54 for 24 hours in the absence or presence of TUDCA. Cells were then washed, and fixed with
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56 4% paraformaldehyde. Then, cells were treated with 5% bovine serum albumin,
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3 permeabilized by 0.1% Triton, incubated with anti-ATF6 (Abcam) for 1 hour, followed by 1
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5 hour incubation with Alexa fluo-546 secondary antibody (ThermoFisher Scientific) at room
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7 temperature. Finally, DAPI (300 mmol/l, Santa Cruz Biotechnology) was added for 5
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9 minutes. Cells were washed and fluorescence was measured with a confocal microscopy
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11 (CLMS 700, Zeiss, ZEN software). All images were acquired using x40 objective.

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14 *Quantitative and semi-quantitative PCR.* Cells were treated for 6 hours as described above
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16 and then, frozen cell pellets were used to investigate the expression of mRNA for CHOP by
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18 RT-PCR (human forward primer GAACGGCTCAAGCAGGAAAT, human reverse primer
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20 TTCACCATTCGGTCAATCAGAG). Total RNA was isolated using miRNeasy Qiagen
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22 microkit (Hilden, Germany). cDNA was generated using PrimeScript™ RT reagent Kit
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24 (Takara/Clontech, Mountain View, CA) from 500 ng of RNA with random hexameric
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26 primers. RT-PCR analyses were performed using a CFX96™ Real-Time PCR Detection
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28 System and SYBR Green detection (Bio-Rad). Semi-quantitative PCR was made for the
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30 expression of XBP-1 (human forward primer AAACAGAGTAGCAGCTCAGACTGC,
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32 human reverse primer TCCTTCTGGGTAGACCTCTGGGAG) by using PTC-200 (MJ
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34 Research, St Bruno, Canada). β -Tubulin was used as the house keeping reference gene.

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38 *siRNA transfection.* Transient transfection of HAoECs was done according to the
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40 manufacturer's protocol (Santa Cruz Biotechnology). Briefly, HAoECs were treated with
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42 either control siRNA or neutral SMAse siRNA for 6 hours in a serum- and antibiotic-free
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44 medium. Cells were then washed and normal fresh medium was added for 24 hours before
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46 treatment with MPs for 24 hours.

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49 *NO measurement.* After treatment for 24 hours, as described above, cells were loaded with 4,
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51 5-diaminofluorescein diacetate (DAF-2DA, Santa Cruz Biotechnology) (10 μ mol/l) for 30
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53 minutes at 37°C, and fluorescence was measured.
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3 *Endothelial function.* Aortic rings were obtained from male Swiss mice bred at the animal
4 facility at the University of Angers. After 24 hours of MP incubation, aortic rings were
5 mounted on a wire myograph filled with physiological salt solution, as previously described
6 (20). Endothelium-dependent vasodilatation was studied by cumulative application of
7 acetylcholine (1 nmol/l-10 μ mol/l, Sigma-Aldrich) in aortas with functional endothelium
8 precontracted with U46619 (Sigma-Aldrich).
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16 *Anion superoxide determination by EPR studies.* The detection of anion superoxide
17 production was performed by EPR technique using 1-hydroxy-3-methylcarbonyl-2, 2, 5, 5
18 tetramethyl pyrrolidine (CMH) (Noxygen, Mainz, Germany) as spin trap. Briefly, after 24
19 hours of treatment, the medium was replaced with 500 μ l of deferoxamine-chelated Krebs-
20 Hepes solution containing CMH (500 μ mol/l), deferoxamine (25 μ mol/l, Sigma-Aldrich), and
21 sodium diethylthiocarbamate (5 μ mol/l, Sigma-Aldrich), and incubated for 45 minutes at
22 37°C. ROS measurement was performed on a table-top x-band spectrometer Miniscope
23 (MS200; Magnetech, Berlin, Germany). Recordings were made at 77°K, using a Dewar
24 flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation,
25 100 kHz of modulation frequency, 150 seconds of sweep time and three scans. The
26 quantitative measurement of the O₂⁻ signal amplitude was reported to the relative units for
27 protein concentration (amplitude/ μ g/ μ l).
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42 *ROS measurement by dihydroethidium (DHE) or MitoSox staining.* Endothelial cells were
43 grown on glass slides (Ibidi), and after different treatments, they were washed and then
44 incubated with either the oxidative fluorescent dye DHE (5 μ mol/l, Sigma-Aldrich) for 30
45 minutes at 37°C or MitoSox red (5 μ mol/l, ThermoFisher Scientific) for 10 minutes at 37°C.
46 Then, cells were fixed by 4% paraformaldehyde for 20 minutes at room temperature. DAPI
47 was added for 5 minutes. Finally, cells were visualized with confocal microscopy (CLMS
48 700, Zeiss, ZEN software). All images were acquired using an x63 objective.
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3 *Measurement of whole cell respiration.* Respiration rates were measured in treated HAoECs.
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5 The cells were collected by trypsinization, washed once in culture medium and centrifuged
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7 (500g, 5 minutes). The pellet was resuspended in respiratory medium, corresponding to
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9 normal culture medium without FBS and antibiotics, both reagents known to interfere with
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11 mitochondrial respiration. Mitochondrial oxygen consumption was measured at 37°C using a
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13 high-resolution Oxygraph-2K respirometer (Oroboros, Innsbruck, Austria). Oxygraphy
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15 experiments were performed on cells cultured in T75 flasks, corresponding to an average of
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17 5×10^6 cells per flask. Basal respiration rate of cells was determined by measuring the linear
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19 rate of oxygen consumption. Oligomycin (1 $\mu\text{g/ml}$) was then added to determine the non-
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21 phosphorylating respiration rate. The uncoupling respiration rate was also recorded by
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23 stepwise addition of FCCP (0.2-1 $\mu\text{mol/l}$) up to the optimal concentration representing the
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25 maximal capacity of the respiratory chain without the regulation of ATP synthase. Finally, the
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27 non-mitochondrial oxygen consumption rate was determined by adding antimycin A (1
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29 $\mu\text{g/ml}$). At the end of the experiment, a standard volume of respiratory medium is collected
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31 and centrifuged for 2 minutes at 16,000g. The cell pellet was then resuspended in NaCl
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33 (0.9%) and protein concentration was determined spectrophotometrically. Respiration rates
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35 were expressed in nmoles of O_2 consumed per minute and per mg protein.
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40 *Mitochondrial enzyme activity measurements.* The activity of the different mitochondrial
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42 respiratory chain complexes was measured on cell homogenates, at 37°C, using a SAFAS
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44 spectrophotometer (SAFAS, Monaco, France).
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47 *Statistics.* Data are expressed as mean \pm SEM. Statistical analyses were performed by a one-
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49 way ANOVA and then Mann-Whitney U, or ANOVA for repeated measures and subsequent
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51 Bonferroni post hoc test. $P < 0.05$ was considered to be statistically significant.
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List of Abbreviations

ADP: adenosine diphosphate

ATF6: activating transcription factor 6

CMH: 1-hydroxy-3-methylcarbonyl-2, 2, 5, 5 tetramethyl pyrrolidine

DHE: dihydroethidium

EDTA: ethylenediaminetetraacetic acid

eIF2 α : Eukaryotic translation initiation factor 2 α

eNOS: endothelial nitric oxide synthase

EPR: electronic paramagnetic resonance

ER: endoplasmic reticulum

FasL: Fas Ligand

HAoECs: human aortic endothelial cells

HRP: horseradish peroxidase

IRE1 α : inositol-requiring enzyme 1 alpha

LDL-R: low density lipoprotein receptor

L-NA: N ω -nitro-L-arginine

MAM: mitochondria-associated membranes

Mfn2: mitofusin 2

MPs: microparticles

NO: nitric oxide

PERK: pancreatic endoplasmic reticulum kinase

PFP: platelet-free plasma

PGC1: peroxisome proliferator-activated receptor gamma coactivator 1

ROS: reactive oxygen species

SMAse: sphingomyelinase

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3 TUDCA: sodium tauroursodeoxycholate
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5 UPR: unfolded protein response
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7 VDAC1: voltage-dependent anion channel 1
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9 XBP1: x-box binding protein 1
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3 **Figure 1: T lymphocytic microparticles (MPs) induce endoplasmic reticulum (ER) stress**
4 **response.** Effects of MPs on the three canonical arms of ER stress response. TUDCA was
5 used to inhibit ER stress response. (A-C) Anti-phospho-PERK, anti-phospho-eIF2 α , anti-
6 CHOP and anti- β actin antibody immunoblots of human aortic endothelial cells. (D)
7 Semiquantitative PCR of XBP-1. (E) Immunofluorescent staining showing the nuclear
8 translocation of ATF6. (F) Nitric oxide (NO \cdot) production measured by electronic
9 paramagnetic resonance. (G) Endothelial-dependent vasorelaxation induced by acetylcholine
10 (Ach) on mouse aortic rings. *P<0.05, **P<0.01. For statistical analyses, one-way analysis of
11 variance and Mann-Whitney U or analysis of variance for repeated measures and subsequent
12 Bonferroni post hoc test for acetylcholine-induced vasorelaxation were used. *n*=3-4.
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26 **Figure 2: ER stress response induced by T lymphocytic microparticles (MPs) is**
27 **mediated by neutral sphingomyelinase (SMase) activation via Fas/Fas-Ligand**
28 **interaction and LDL-receptor.** (A-C) Effects of MPs on ER stress response (phospho-
29 eIF2 α) and Smase expression are inhibited by anti-Fas-Ligand or LDL-receptor antibodies.
30 (D-H) Pharmacological inhibition of SMase with GW4869 or silencing with siRNA abolished
31 MP-induced ER stress response and endothelial dysfunction. *P<0.05. For statistical analyses,
32 one-way analysis of variance and Mann-Whitney U were used. *n*=3-4.
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42 **Figure 3: Cytosolic production of reactive oxygen species by MPs is upstream of ER**
43 **stress response.** (A-B) Effects of MPs on ER stress response (phospho-eIF2 α) are inhibited
44 by inhibitors of ROS sources. (C-I) Cytosolic ROS production measured by electronic
45 paramagnetic resonance (C-E) and DHE labeling (F-I). (J) Effects of inhibitors of ROS
46 sources on ROS production after 4 hours of MP treatment. (K) Effects of silencing SMase on
47 MP-induced p47phox expression. *P<0.05, **P<0.01, ***P<0.001. For statistical analyses,
48 one-way analysis of variance and Mann-Whitney U were used. *n*=3-7.
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3 **Figure 4: Mitochondrial production of reactive oxygen species by MPs is downstream of**
4 **ER stress response. (A-D)** Mitochondrial ROS production measured by MitoSox labelling.
5 **(E)** Scavenging mitochondrial ROS with Mito-Tempo has not effect on ER stress response.
6 **(F-I)** Effects of MPs on mitochondrial respiration and complex IV activity. **(J and K)** Effects
7 of MPs on mitochondria-associated membranes. **(L)** Mito-Tempo improves NO[•] production
8 induced by MPs. *P<0.05, **P<0.01. For statistical analyses, one-way analysis of variance
9 and Mann-Whitney U were used. n=4-6.

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19 **Figure 5: Microparticles from metabolic syndrome patients (MetS MPs) induce**
20 **endoplasmic reticulum (ER) stress response via Fas/Fas-Ligand interaction and neutral**
21 **sphingomyelinase (SMase) pathway.** Effects of MPs from healthy subjects (HS MPs) and
22 MetS MPs on the canonical arms of ER stress response. TUDCA was used to inhibit ER
23 stress response. **(A)** Anti-phospho-eIF2 α antibody immunoblots of human aortic endothelial
24 cells. **(B)** Immunofluorescent staining showing the nuclear translocation of ATF6. **(C)** Nitric
25 oxide (NO[•]) production measured by electronic paramagnetic resonance. **(D)** Endothelial-
26 dependent vasorelaxation induced by acetylcholine (Ach) on mouse aortic rings. **(E and F)**
27 Effects of MPs on ER stress response (phospho-eIF2 α) and SMase expression are inhibited by
28 anti-Fas-Ligand antibodies. **(G and H)** Silencing SMase with siRNA abolished MP-induced
29 ER stress response. *P<0.05, **P<0.01. For statistical analyses, one-way analysis of variance
30 and Mann-Whitney U or analysis of variance for repeated measures and subsequent
31 Bonferroni post hoc test for acetylcholine-induced vasorelaxation were used. n=3-4.

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49 **Figure 6: Cytosolic and mitochondrial production of reactive oxygen species (ROS) by**
50 **MetS MPs are downstream of ER stress response. (A-D)** Cytosolic ROS production
51 measured by DHE staining. **(E-H)** Mitochondrial ROS production measured by MitoSox
52 labelling. For statistical analyses, one-way analysis of variance and Mann-Whitney U were
53 used. n=4.

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3 **Figure 7: neutral sphingomyelinase controls p47phos expression.** siRNA against neutral
4 sphingomyelinase (SMAse) prevents on p47phox expression induced by MetS MPs at 4 hours
5 (A), but not at 24 hours (B). *P<0.05. For statistical analyses, one-way analysis of variance
6 and Mann-Whitney U were used. n=4.
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12 **Figure 8: Schematic overview of effects of T lymphocytic microparticles (MPs) (A) and**
13 **MPs from metabolic syndrome patients (MetS MPs) (B) on endothelial cells. (A)** MPs
14 from apoptotic T cells act on Fas and LDL-receptors, then, via neutral SMase cascade induce
15 cytosolic ROS production which activates ER stress. Through the interaction between ER and
16 mitochondria, mitochondrial ROS are increased and contribute to decreased bioavailability of
17 NO[•] and the subsequent impairment of endothelium-dependent vasorelaxation. **(B)** Fas/FasL,
18 but not LDL-receptor, participates in the response induced by MetS MPs. In addition, neutral
19 SMase activation induces directly ER stress which, in turn, increases both cytosolic and
20 mitochondrial ROS. All of these events lead to reduction of NO[•] release and the subsequent
21 impairment of endothelium-dependent vasorelaxation.
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Figure 1

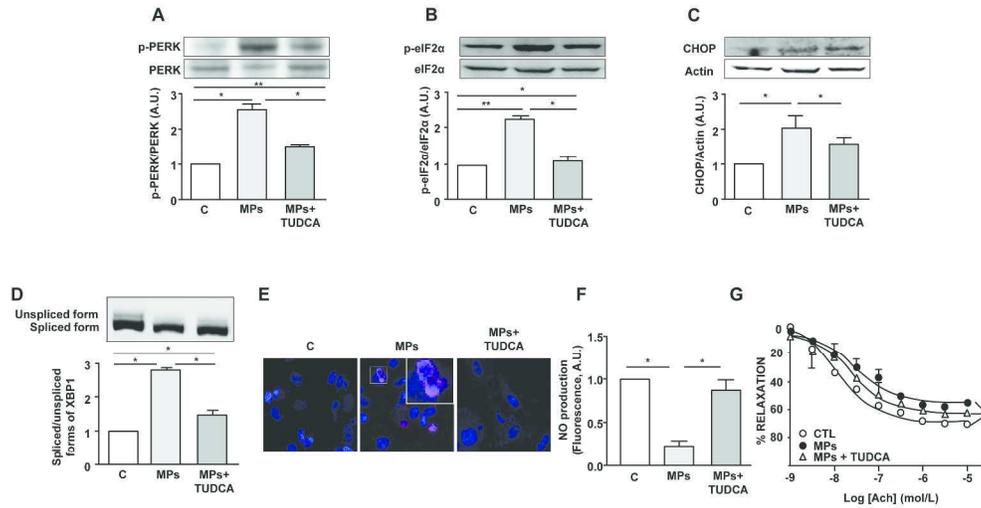
Figure 1
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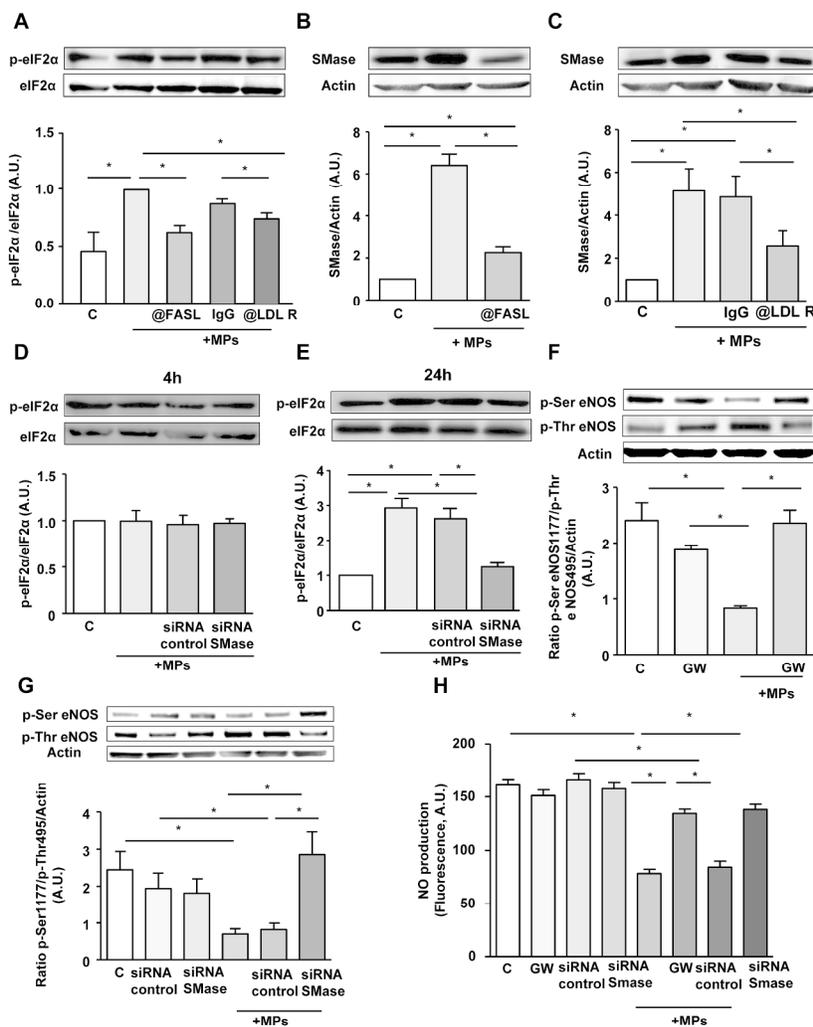


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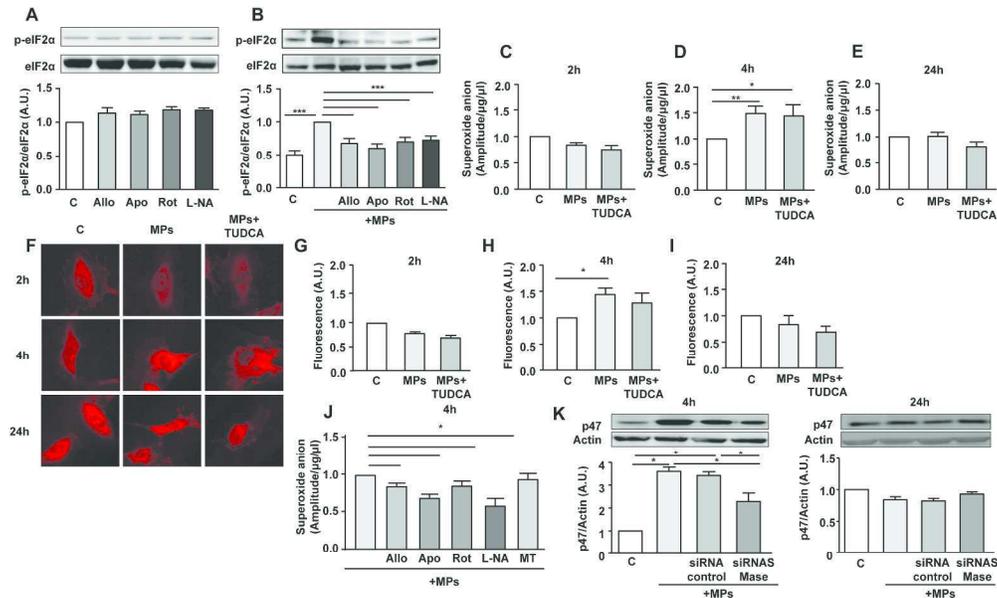
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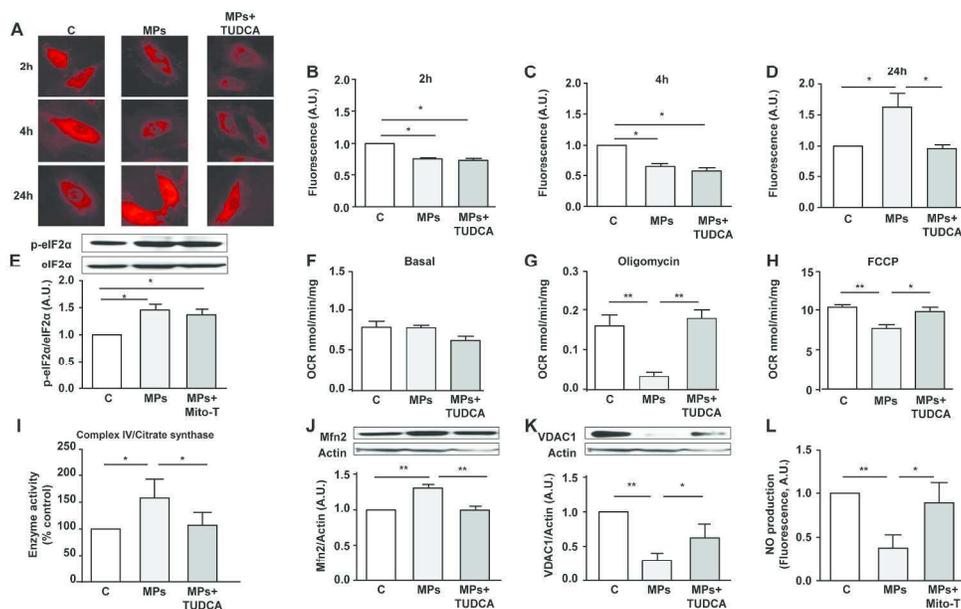


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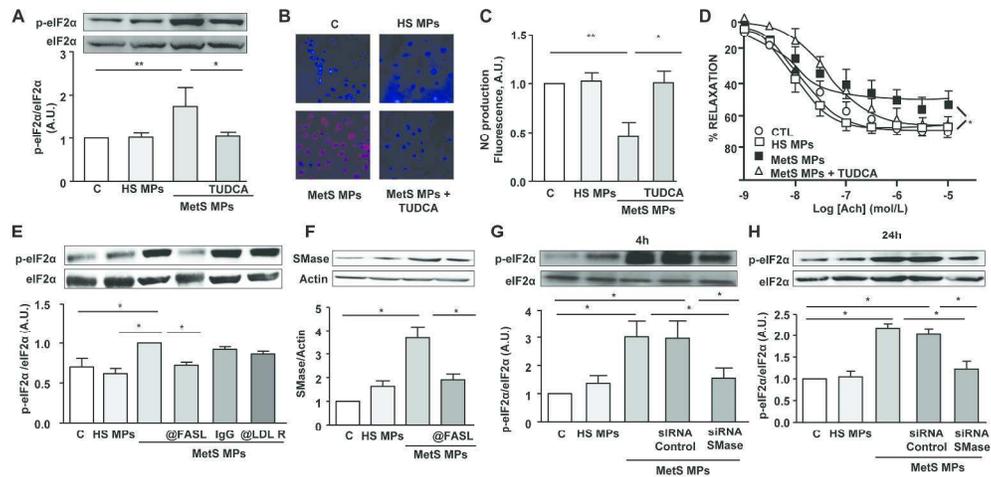
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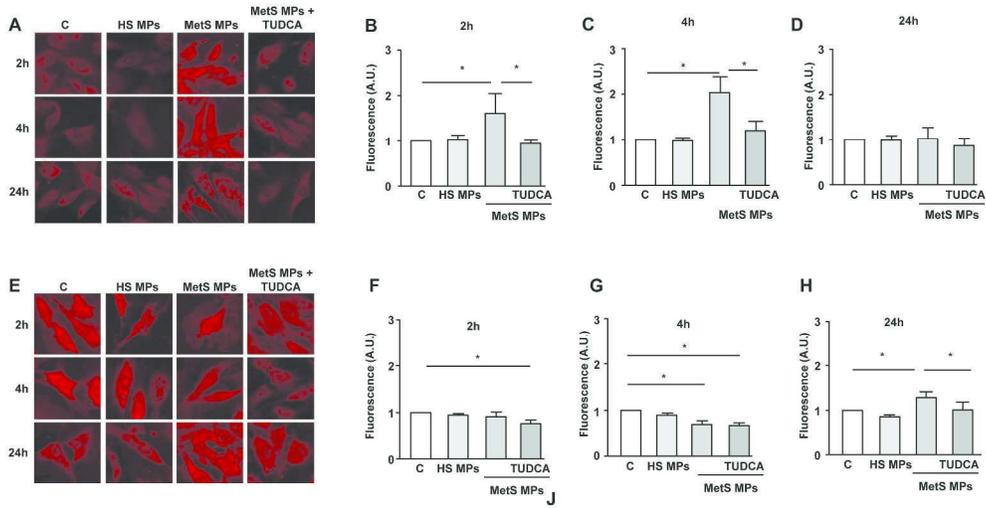


Figure 6
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Figure 7

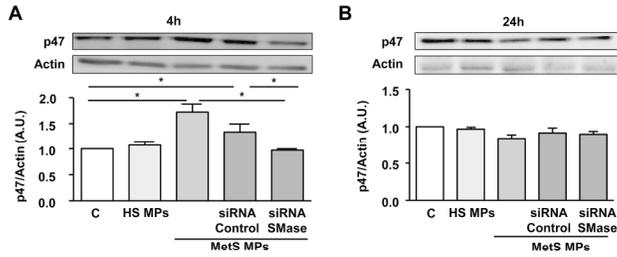


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Figure 7

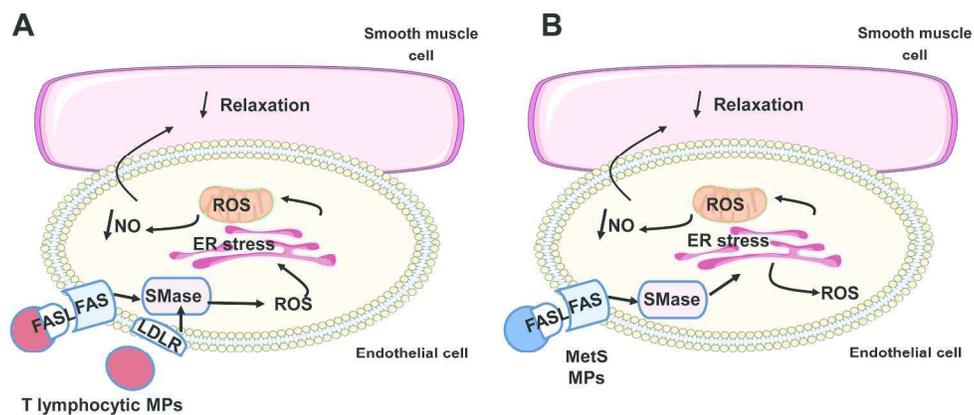


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Supplementary Table 1. Baseline characteristics of metabolic syndrome patients and healthy subjects.

	Healthy subjects	Metabolic syndrome patients
Number	20	30
Mean age (years)	49 ± 3	55 ± 2
Sex ratio (male/female)	12/8	18/12
BMI (kg/m²)	25 ± 1	33 ± 1***
Waist circumference (cm)	89 ± 3	114 ± 3*
Systolic blood pressure (mmHg)	122 ± 3	126 ± 3
Diastolic blood pressure (mmHg)	78 ± 2	73 ± 2
Glycemia (g/l)	0.95 ± 0.02	1.31 ± 0.16
HbA1c (% of total Hb)	6.04 ± 0.36	6.17 ± 0.11
Total cholesterol (g/l)	1.34 ± 0.18	2.17 ± 0.11***
HDL cholesterol (g/l)	0.56 ± 0.04	0.46 ± 0.03*
LDL cholesterol (g/l)	0.96 ± 0.07	1.23 ± 0.07*
Triglycerides (g/l)	1.03 ± 0.16	2.11 ± 0.36*
Number of metabolic syndrome components (%)		
0	52	
1	29	
2	19	
3		41
4		41
5		18
Treatment (%)		
Oral antidiabetic	14	34
Antihypertensive	19	52
Statins	5	27

Supplementary Table 2. Circulating microparticle levels in patients with metabolic syndrome compared to healthy subjects. Total MP levels and different subsets of MPs platelet- (CD41⁺), endothelial- (CD146⁺), erythrocyte- (CD235⁺), leukocyte- (CD45⁺) derived and procoagulant (annexin V⁺) microparticles.

	Healthy subjects	Metabolic syndrome patients
Total microparticles/ μ l plasma	31,450 \pm 7,932	75,370 \pm 23,510 ^{***}
CD41 ⁺ microparticles/ μ l plasma	26,210 \pm 5,049	64,820 \pm 18,480 ^{**}
CD146 ⁺ microparticles/ μ l plasma	274 \pm 118	407 \pm 193 ^{***}
CD235 ⁺ microparticles/ μ l plasma	627 \pm 251	3,035 \pm 1,155 ^{***}
CD45 ⁺ microparticles/ μ l plasma	310 \pm 102	834 \pm 210 [*]
Annexin V ⁺ microparticles/ μ l plasma	2727 \pm 872	5,760 \pm 2,504 [*]

Results are expressed as events/ μ l of plasma and given as mean \pm SEM. *P<0.05, **P0.01, ***P<0.001 versus healthy subjects.

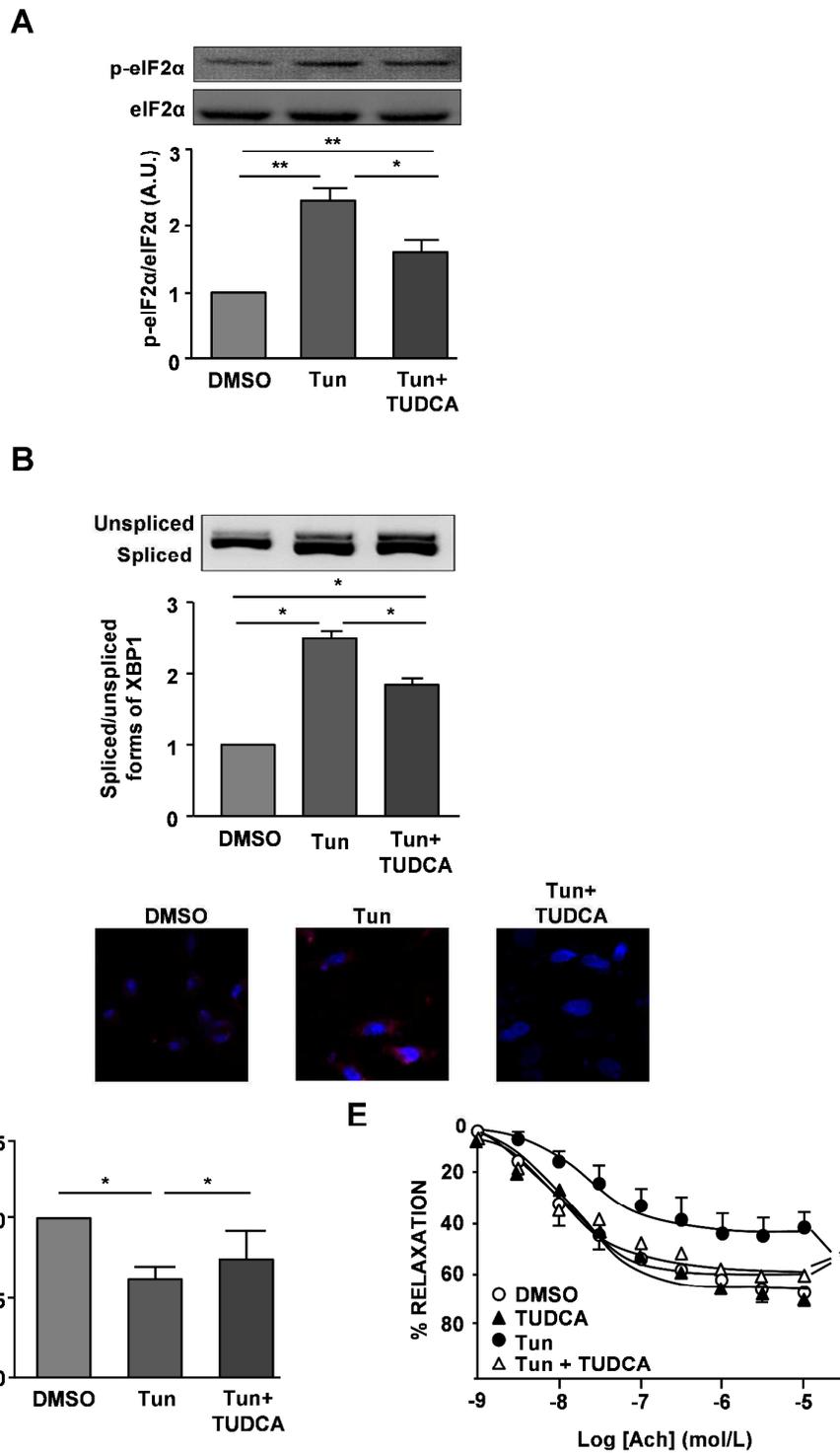


Fig S1

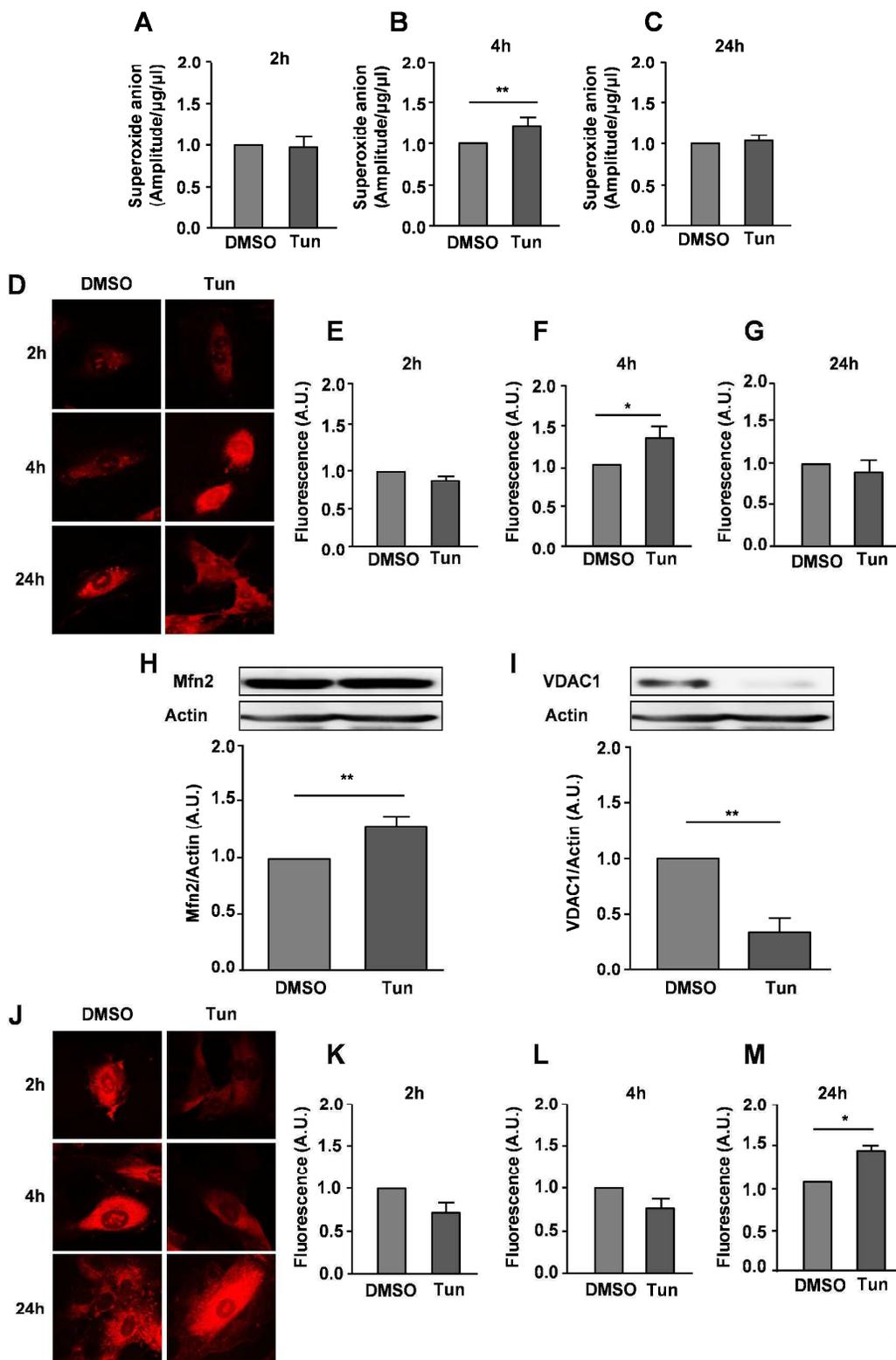


Fig S2

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Legends of Supplemental Figures

Supplemental Figure 1: Tunicamycin (Tun) induces endoplasmic reticulum (ER) stress

response. Effects of Tun on the three canonical arms of ER stress response. TUDCA was used to inhibit ER stress response. **(A)** Anti-phospho-eIF2 α antibody immunoblots of human aortic endothelial cells. **(B)** Semiquantitative PCR of XBP-1. **(C)** Immunofluorescent staining showing the nuclear translocation of ATF6. **(D)** Nitric oxide (NO) production measured by electronic paramagnetic resonance. **(E)** Endothelial-dependent vasorelaxation induced by acetylcholine (Ach) on mouse aortic rings. *P<0.05, **P<0.01. *n*=3-4.

Supplemental Figure 2: Cytosolic and mitochondrial production of reactive oxygen

species by tunicamycin (Tun) is downstream of ER stress response. **(A-G)** Cytosolic ROS production measured by electronic paramagnetic resonance (A-C) and DHE labeling (D-G). **(H and I)** Effects of Tun on mitochondria-associated membranes. **(J-M)** Mitochondrial ROS production measured by MitoSox labelling. *P<0.05, **P<0.01. *n*=3-7.

DISCUSSION & CONCLUSION

This thesis work explained the pathway through which the two types of MPs, those *in vitro* generated from apoptotic T cells, and those collected from MetS persons drive endothelial dysfunction. Both kinds of MPs caused the activation of the ER stress leading to endothelial dysfunction although they did not implicate the same mechanisms (Figure 9). Indeed, although both types of MPs are able to activate Fas, LDL receptor is only implicated in the effects evoked by T apoptotic MPs. Furthermore, once the surface receptors on endothelial cells were activated, they converge on the overexpression of NSMase. From that moment, the pathways are diverging. Indeed, although ER stress response and cytosolic and mitochondrial ROS production are obligatory for MP-induced endothelial dysfunction, the spatial-temporal ER stress and ROS production were different.

MPs were found to interact with endothelial cells either through Fas/FasL (case of MetS MPs) or through Fas/FasL and LDL receptor (case of apoptotic T MPs). Agouni et al ⁽⁵¹⁾ has shown that the vascular effects of MetS MPs may be mediated by the interaction of FasL, harbored by MetS MPs, and Fas receptor expressed by smooth muscle cells. Interestingly, endothelial cells express a little amount of Fas compared to vascular smooth muscle cells; however, the expression of Fas can be upregulated by the presence of FasL. Of note, Sata and colleagues showed that endothelial cells did not undergo apoptosis after the infection with Adeno-FasL ⁽¹³⁴⁾ according with the results of the present Thesis where apoptosis was not found at 24 hours of MP treatment.

Findings provided evidence that stimulation of cells with anti-Fas antibodies induces the activation of NSMase and the generation of ceramide ⁽¹³⁵⁾, however, ceramide production has been associated in many, but not all, cases with apoptosis ^(136, 137). Lin T et al showed that SMase activation through Fas engagement results in ER stress activation. This is in agreement with the results of the present study, indicating that interaction of Fas/FasL activates NSMase. On the other hand, MPs from apoptotic T cells interacted with endothelial cells through Fas/FasL as well as with LDL receptor. Yang et al ⁽⁷⁹⁾ indicated that MPs from Jurkat T cells interacted with human retinal endothelial cells via LDL receptor-mediated endocytosis.

The two receptors turned on SMase. In fact, when NSMase was silenced or pharmacologically inhibited all downstream events of the cascade were prevented suggesting that NSMase is a key actor of the signaling leading to the endothelial dysfunction. It should to be noted that the

inhibitor of the acid SMase D609 had no effect (results not shown) reinforcing the notion that in our study NSMase, but not acid SMase, was implicated in the effects of MPs.

Several hypotheses can be advanced in order to explain the observed differences. The composition and content of the MPs might be the reason beyond this difference in the track of signalization. Thus, whereas MPs generated by *in vitro* treatment of T lymphocytes are homogeneous in composition, MPs from patients are heterogeneous since they are generated from different cell types (platelets, endothelial cells, erythrocytes and leukocytes). In this respect, protein composition of MPs from MetS patients has been compared with those from healthy subjects (unpublished results). Nine proteins were differentially expressed and it is plausible that they can participate to the different activated pathways in endothelial cells.

Another possible explanation is that both types of MPs activate NSMase at the different degree leading to increase ceramide levels in a dose-dependent manner which is associated with increased ROS production. This may be verified by quantification of ceramide level produced by MP treatment.

Since the effects induced by MPs on endothelial cells were not modified in the presence of RNase, this means that they are not mediated by the miRNA or mRNA that MPs transport. However, it is possible that different ceramide production can regulate miRNA levels and subsequently modulate ROS production as a primary effect of action, such as in the case of miR155 in endothelial cells ⁽¹³⁸⁾. At their turn, p47 phox (cytosolic regulatory subunit of NADPH oxidase) and ROS are found to modulate the expression of miRNAs, which might lately be involved in ER stress induction ⁽¹³⁹⁾. Maurel et al ⁽¹⁴⁰⁾ explained the existence of a miRNA network that constitutes an additional layer in the regulation of the ER stress-induced UPR. Not only, many stress responses that perturb the ER have been linked to miRNAs, but also the integration of miRNAs into specific UPR signaling pathways has only recently emerged ⁽¹⁴¹⁾, even though the three canonical arms of the UPR have been activated in our model. Indeed, it is now apparent that ER stress-regulated miRNAs may modulate translation of UPR effectors, synthesis of secretory pathway proteins and the fate of cells challenged with increased demands on the ER ⁽¹⁴²⁾, in other words miRNAs contributes to the bi-functional, pro-adaptive/pro-apoptotic roles of the UPR ⁽¹⁴¹⁾.

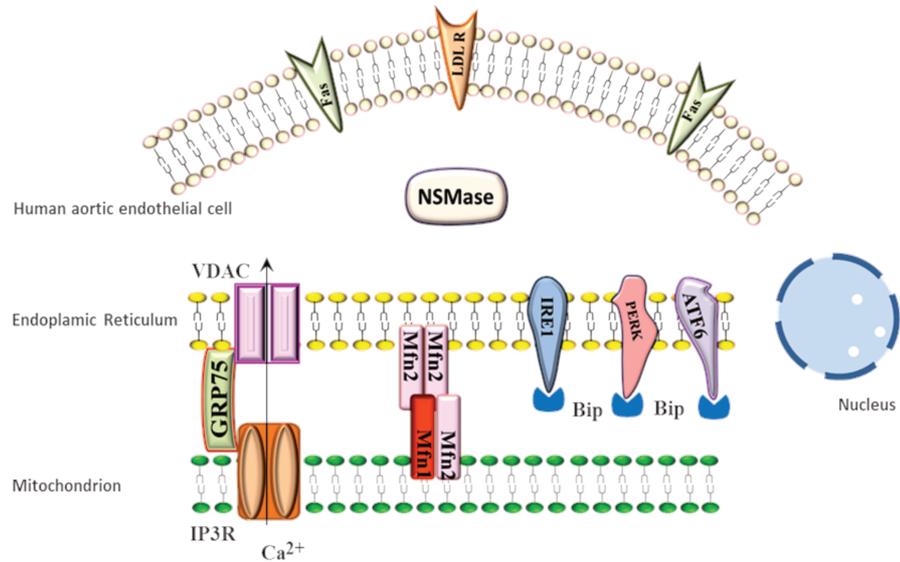
MPs induce changes in the MAMs where upon MP treatment we had an increase in Mfn2. Wang et al showed that Mfn2 overexpression blocks mitochondrial fragmentation and dysfunction, and/or neuronal death in spinal cord motor neurons both *in vitro* and in mice ⁽¹⁴³⁾. At a certain level, VDAC expression was found to be down regulated. Studies showed that increased expression of VDAC1 seemed to augment the sensitivity to the ER-stress and

increased VDAC1 expression sensitizes myeloma cells to the various extracellular stimuli that trigger apoptosis via the mitochondrial pathways⁽¹⁴⁴⁾. These results suggest that the changes in the mitochondria might be a sort of self-protection at the small level and ways for cell rescue at a bigger level.

Finally, ER stress activation along with increased cytosolic ROS production led to mitochondrial dysfunction accompanied with increased mitochondrial ROS accounting for the endothelial dysfunction. Li et al⁽¹³³⁾ showed that, under stress conditions and increased ROS production, mitochondrial ROS generation increases causing mitochondrial dysfunction and causes eNOS uncoupling resulting in endothelial dysfunction.

Nevertheless, although the mechanisms involved in the endothelial dysfunction induced by human MPs are sensibly different, they lead to the same end-point, a reduction of NO production which is, at least in part, responsible of the alteration of endothelial function. This work highlights several new targets (NSMase, UPR and mitochondria) that deserve to be further studied in order to reduce endothelial injury evoked by MPs.

Normal Conditions



Treatment with MPs

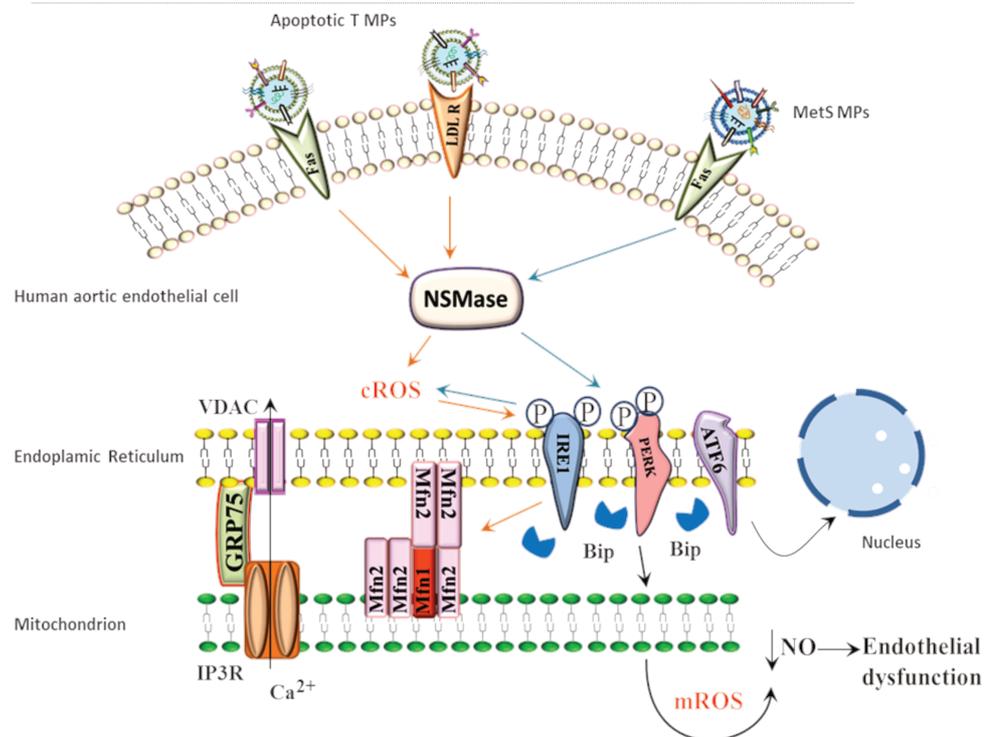


Figure 9. The pathways taken by apoptotic T MPs and MetS MPs in inducing endothelial dysfunction. Apoptotic T MPs interact through Fas/FasL and LDL receptor with human aortic endothelial cells, whereas MetS MPs act on endothelial cells solely through Fas/FasL. Both types of MPs activate NSMase which in turn either increases cROS production, that activates ER stress (apoptotic T MPs), or runs ER stress that causes the generation of cROS (MetS MPs). Eventually, activated ER stress and increased cROS production, increases Mfn2 and decreases VDAC expression (apoptotic T MPs) and causes the generation of mROS (apoptotic T MPs & MetS MPs), driving at the end an endothelial dysfunction.

REVIEW



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Organelles in focus

Dialogue between endoplasmic reticulum and mitochondria as a key actor of vascular dysfunction associated to metabolic disorders

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ABSTRACT

Metabolic syndrome due to its association with increased risk of cardiovascular diseases and cardiac mortality, comprises a cluster of metabolic abnormalities such as central obesity, hyperglycemia, dyslipidemia, and hypertension. Recent studies have shown that metabolic syndrome patients exhibit impaired nitric oxide-mediated vasodilatation leading to endothelial dysfunction in addition to insulin resistance. Interestingly, development and maintenance of the unfolded protein response of the endoplasmic reticulum stress revealed a surprisingly direct link with metabolic syndrome and endothelial dysfunction. On the other hand, in metabolic disorders, interaction between endoplasmic reticulum and mitochondria is mandatory for the generation of mitochondrial oxidative stress and perturbation of mitochondrial function accounting, at least in part, for vascular dysfunction. Herein, we review the impact of the dialogue between endoplasmic reticulum and mitochondria in modulating the cellular signals governing vascular alterations associated to metabolic disorders.

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1. Metabolic disorders as risk factors of cardiovascular diseases

Metabolic disorders including insulin resistance, diabetes, dyslipidemia and obesity are considered as risk factors involved in the increase to develop cardiovascular diseases (CVD) (Kaur, 2014). In particular, patients with metabolic disorders are more exposed to atherosclerosis, ischemic stroke (Ballantyne et al., 2008) and myocardial infarction (Deedwania, 2004) than healthy subjects. Among the changes at the vascular level affecting cardiovascular dysregulation, endothelial dysfunction is implicated in the initiation and progression of metabolic disorders (Ahirwar et al., 2015) and, on the other hand, it represents an early step for CV events (Totoson et al., 2014). Individuals with metabolic disorders exhibit higher degree of endothelial dysfunction (Kwaśniewska et al., 2015) probably due to the reduced plasmatic levels of nitric oxide (NO) in these patients (Ahirwar et al., 2015) as well as enhanced reactive oxygen species (ROS) generation, inflammation and changes of barrier function (Bakker et al., 2009). More

precisely, the association of increased visceral obesity and other metabolic perturbations leading to oxidative stress and chronic inflammation impairs NO bioactivity, causing endothelial dysfunction (Kwaśniewska et al., 2015; Li et al., 2012). Furthermore, insulin resistance may impair NO bioavailability (Kwaśniewska et al., 2015) and decrease PI3 K/Akt signaling in the vascular wall, thus issuing endothelial damage.

2. Regulation of the endothelial function by the endoplasmic reticulum (ER) and mitochondria

Accumulating evidence suggests that dysregulation of ER and/or mitochondria actively participate in the development and maintenance of pathophysiological states, including metabolic diseases. In this part of the review, we will analyze the potential role of these organelles in the pathogenesis of vascular complications of metabolic disorders focusing particularly on their participation in the endothelial dysfunction. Endothelial cell layer is specially targeted in metabolic alterations due to its strategical localization. Indeed, endothelial cells are directly in contact with elevated concentration of glucose, insulin and triglycerides in blood suggesting that endothelial function alterations play an early stage in CVD. Endothelial dysfunction is related to decreased NO production in addition to endothelial-derived hyperpolarizing factor, increased

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expression of adhesion and pro-inflammatory molecules and exacerbated ROS production.

2.1. ER and endothelial dysfunction

The ER is the organelle responsible for protein folding and maturation (Ron and Walter, 2007) and, among its main functions; ER participates to Ca^{2+} storage, lipid and glucose metabolism, and signal transduction (Tripathi and Pandey, 2012). The unfolded protein response (UPR) expresses the ER-to-nucleus signaling cascades, which supervises ER homeostasis (Qi et al., 2011). The UPR represents the imbalance between the amount of unfolded proteins entering the ER and the ability of the cellular machinery to deal with that amount (Ron and Walter, 2007). In metabolic disorders, this is mainly due to nutrient overload, along with increased demand for protein synthesis for its metabolism and local glucose deprivation due to insulin resistance (Achard and Laybutt, 2012). Besides, depending on tissues, UPR is triggered by a variety of pathological factors, such as nutrient deprivation, altered glycosylation, calcium depletion, oxidative stress, DNA damage and energy disturbance (for review see Liu et al., 2016). Three distinct classes of ER stress transducers that determine three different arms of the UPR have been determined: the inositol-requiring protein-1 (IRE1), the protein kinase RNA (PKR)-like ER kinase (PERK), and the activating transcription factor-6 (ATF6) (Ron and Walter, 2007). ER luminal chaperons, especially the binding immunoglobulin protein/glucose regulated protein 78 BIP/GRP78 of the HSP70 class, keep the former ER stress sensors inactive. Upon the accumulation of unfolded proteins, BIP is isolated from the ER stress sensors luminal domain, leading to their activation to reestablish homeostasis (Schröder and Sutcliffe, 2010). Interestingly, the link between ER stress and metabolic diseases is well established (for review see Tripathi and Pandey, 2012). Genetic overexpression of the ER chaperones in obese/diabetic mice has been shown to significantly improve insulin resistance and ameliorate glucose tolerance (Nakatani et al., 2005). Also, treatment with chemical ER chaperones like tauroursodeoxycholate (TUDCA), an ER stress inhibitor, improves metabolic disturbances reflected by improvement of insulin sensitivity in obese humans (Kars et al., 2010).

Growing evidences support a critical role of ER stress in the development of the endothelial dysfunction. In a streptozotocin-induced hyperglycemic ApoE^{-/-} mouse model, hyperglycemia promotes overexpression of endothelial ER stress markers in the aorta wall before any morphologic changes in vessel structure or cellular organization. Furthermore, the accelerated development of atherosclerotic lesions occurs before the onset of diabetes-associated dyslipidemia suggesting that ER stress takes place at an early stage of diabetes (Khan et al., 2009). Interestingly, oxidized LDLs through ROS production induce ER stress response in human endothelial cells (Sanson et al., 2009). Furthermore, at the tissue levels, these authors have also described the expression of ER stress markers in advanced atherosclerotic lesions (Sanson et al., 2009). Accordingly, endothelial XBP1 expression is found in areas of lesion severity, and atherosclerosis is quickened in lesions overexpressing XBP1, in ApoE^{-/-} mice suggesting that high XBP1 levels damages the cells and therefore boosts atherosclerosis (Khan et al., 2009; Zeng et al., 2009). At the cellular level, treatment with tunicamycin, an ER stress inductor, of human aortic endothelial cells induces increased secretion of pro-inflammatory cytokines such as IL6, IL8, monocyte chemoattractant protein 1 (MCP-1), and the chemokine CXC motif ligand 3 (CXCL3), which play a role during atherogenesis (Gargalovic et al., 2006). Galán et al. (2014) have shown that *in vivo* treatment with tunicamycin leads to ER stress response which is associated with vascular endothelial dysfunction assessed by acetylcholine relaxation (Galán et al., 2014). Conversely, *in vivo* inhibition of ER stress with TUDCA not

only improves ischemia-induced neovascularization by increasing pro-angiogenic pathways (endothelial NO-synthase (eNOS) and VEGFR2 signaling) but also normalized glucose, insulin and cholesterol levels in diabetic mice (Amin et al., 2012) suggesting that ER stress response contributes to metabolic complications in diabetes. Altogether, these data highlight the critical role of ER stress response in the development of endothelial dysfunction.

2.2. Mitochondria and endothelial dysfunction

Mitochondria are essential organelles involved in the pathophysiology of obesity and its vascular complications (for review see Duluc et al., 2012). These deleterious effects occur mainly through the mitochondrial biogenesis, dynamics and oxidative phosphorylation (OXPHOS) dysfunction leading to ROS production and apoptosis. These effects account for loss of endothelium integrity. Although mitochondrial content in endothelial cells is relatively low compared to cardiomyocytes and hepatocytes (Tang et al., 2014), mitochondria play a key role on endothelial function. Indeed, it has been shown that overexpression of PGC-1 α , a factor regulating mitochondrial biogenesis, protects against oxidative stress and limits atherosclerotic lesion formation (Stein et al., 2010). Moreover, altered expression of components of the OXPHOS chain is related to impaired mitochondrial biogenesis that may contribute to endothelial senescence (Ungvari et al., 2008). Also, mitochondrial fragmentation and the subsequent loss of mitochondrial networks have been described in endothelial cells from diabetic patients as well as in cultured human aortic endothelial cells exposed to high glucose concentration (Shenouda et al., 2011). This was associated with the impairment of the eNOS activity and enhanced ROS production. Mitochondrial ROS can damage mitochondrial DNA, lipids and proteins as well as the complexes forming the OXPHOS chain that in turns can contribute to the self-perpetuating mitochondrial ROS production. Also, in a murine model of obesity, high-fat diet induces mitochondrial fragmentation along with decrease in mitofusin 2 (Mfn2) levels, a key component of mitochondrial fusion (Jheng et al., 2012; Schneeberger et al., 2013). Moreover, high levels of glucose lead to mitochondrial fragmentation associated with increased ROS production (Schneeberger et al., 2013). In addition, obese humans and type 2 diabetes patients display low Mfn2 transcript levels. Interestingly, upon acute weight loss these levels are increased, this raises the concept that nutrient status can adjust mitochondrial dynamics (Bach et al., 2005).

In summary, mitochondrial dysregulation at different levels is implicated in the pathogenesis of vascular complications observed during metabolic disorders.

3. ER stress and mitochondria dialogue: role on endothelial dysfunction

It is well established that ER and mitochondria collaborate between them through the physiological contact sites contributing for the exchange of metabolites such as lipids and Ca^{2+} between both organelles (Kornmann, 2013). These interconnections called mitochondria-associated membrane (MAMs) participate in the dialogue between ER and mitochondria (Vance, 1990) and play a major role in tethering and saving homeostasis and signaling pathways between the two organelles (Kornmann, 2013) (Fig. 1). For instance, an increase in the number of ER-mitochondria contact sites enhances Ca^{2+} transfer to the mitochondria allowing that cells adapt to stress conditions require enhanced metabolic output (Kopeck et al., 2010). Interestingly, under pathophysiological conditions, emerging data show that contact sites between ER and mitochondria may play a key role in the pathogenesis of metabolic disorders. In this respect, mitochondrial Ca^{2+} overload favors apop-

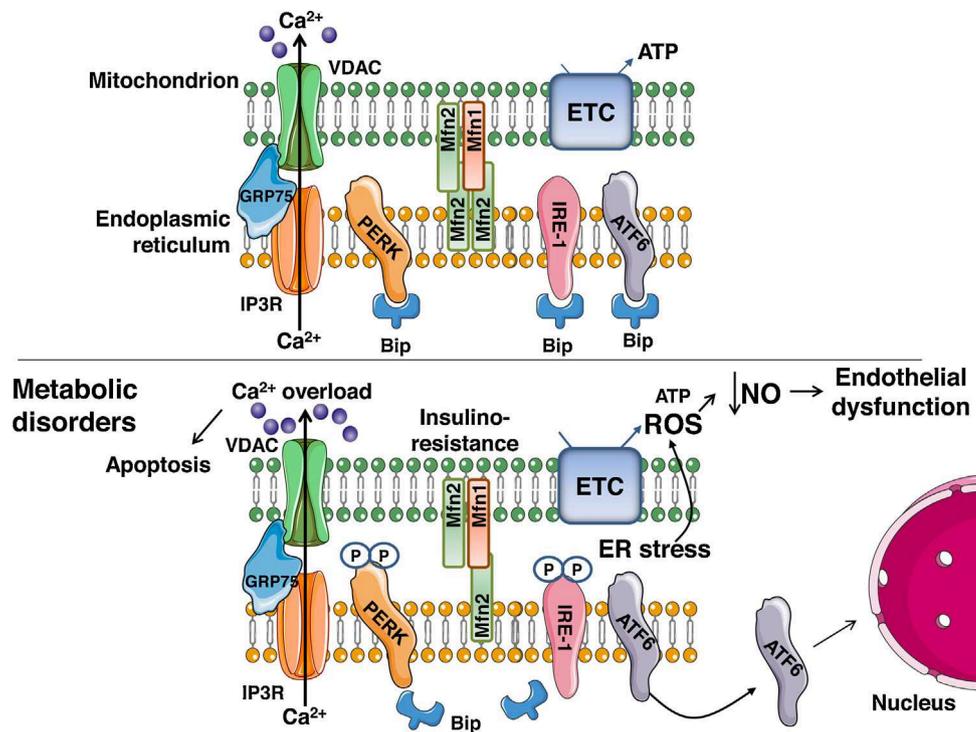


Fig. 1. Dialogue between endoplasmic reticulum (ER) and mitochondria. Under physiological conditions, both organelles interact with each other through different proteins in the specialized region called mitochondria-associated membrane (MAM). Mainly, the complex formed by voltage-dependent anion channel (VDAC)-1/glucose-regulated protein 75 (Grp75)/inositol 1,4,5-triphosphate receptor (IP3R) regulates Ca^{2+} exchanges between ER and mitochondria. Interaction of mitofusin (Mfn) 1 and 2, two proteins related with mitochondria fusion, with ER-bound Mfn2 participates to insulin signaling. In metabolic disorders, excessive Ca^{2+} fluxes from the ER to the mitochondria result in apoptosis. Also, disruption of the interaction of Mfns, is implicated in insulin-resistance. ER stress response due to the activation of the three canonical branches (phosphorylation of PERK and IRE-1 and nuclear ATF6 translocation) induces mitochondrial dysfunction. Indeed, the impairment of the electron transport chain (ETC) leads to reactive oxygen species (ROS) generation that accounts for the reduction of nitric oxide (NO) bioavailability and the subsequent endothelial dysfunction.

totic response (Decuyper et al., 2011). In hepatocytes, the integrity of MAM is necessary for insulin signaling *in vivo*. Indeed, loss-of-function MAM-associated Mfn2 impairs insulin signaling in liver as reflected by defective insulin receptor tyrosine phosphorylation and Akt phosphorylation (Sebastián et al., 2012). In *ob/ob* mice and mice fed with high-fat/high-sucrose diet, MAM integrity is altered in liver and antidiabetic treatment improved communication between ER and mitochondria (Tubbs et al., 2014). Also, in liver of high-fat fed mice, a marked reorganization of MAMs resulting in mitochondrial Ca^{2+} overload has been reported (Arruda et al., 2014). This effect compromised OXPHOS capacity and augmented oxidative stress. In addition, rapid induction of ER stress response with tunicamycin induced changes on MAM indicating that ER stress regulates the dialogue with mitochondria independently of obesity. However, it is not clear whether ER stress response regulates mitochondrial function (Rainbolt et al., 2014), or at the contrary, ER stress response emerges as a result of mitochondrial dysfunction (Kozlov et al., 2009).

Indirect evidence suggests that alteration of ER-mitochondria dialogue may be involved in the endothelial dysfunction described in metabolic disorders. Indeed, in human aortic endothelial cells, ER stress and mitochondria via the Ca^{2+} /calmodulin-dependent protein kinase II are implicated in thapsigargin-induced apoptosis (Timmins et al., 2009). Also, alteration of the structural and spatial mitochondrial network which is associated with ER stress response leads to loss of cerebrovascular homeostasis at the endothelial level (Plácido et al., 2016). In human retinal capillary pericytes, oxidized LDL subsequently triggers sequential oxidative stress, ER stress and mitochondrial dysfunction, with apoptosis and autophagy as final outcomes indicating that ER stress response is responsible of

alterations in mitochondrial function (Fu et al., 2012). In agreement with these data, Lu et al. (2013) have found that high-fat diet rich in saturated fatty acid palmitate induces swollen mitochondria and extends ER in endothelial cells of mouse aorta. These morphological changes are associated with increased ROS production, overexpression of ER stress markers and insulin-resistance and endothelial dysfunction. These data illustrate the collaborating role of both ER and mitochondria in inducing endothelial dysfunction in metabolic disorders. When endothelial cells are exposed to ischemia/reperfusion which is a robust source of ROS, increased Ca^{2+} flux from ER to mitochondria through MAM interactions occurs and disruption of MAM protects endothelial cells from ischemia/reperfusion injury (He et al., 2015). Interestingly, morphology and function of both ER and mitochondria in endothelial cells is dependent of NO. Indeed, it has been shown that reduced NO bioavailability induces tubule-to-sheet change of ER structure, Golgi fragmentation and increased fibrillarity of the mitochondria that is associated with the development of a prosecretory phenotype of endothelial cells (Lee et al., 2013). It is plausible to propose that under pathological conditions in which NO production is reduced, similar changes on the structure of these organelles take place.

4. Future outlook

Although the cross-talk between ER and mitochondria is fairly well studied in metabolic disorders, the veritable implication in the pathogenesis of the vascular consequences of these diseases remains to be exhaustively proved. Clinical trials using inhibitors of ER stress response such as TUDCA or 4-phenylbutyrate show that

short oral treatment (4 or 2 weeks, respectively) improves insulin sensitivity suggesting that inhibition of ER stress response can represent new therapeutic opportunities (Kars et al., 2010; Xiao et al., 2011). However, no evidences of the potential therapeutic effects on the endothelium has been analyzed. The new knowledge of the link between these two organelles will provide basis for the development of upcoming, personalized interventions for the treatment of vascular consequences of metabolic disorders.

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PUBLICATION

Abstract

Recent studies have shown the implication of ER stress in neuropathological diseases such as Parkinson's disease and Alzheimer diseases. Indeed, it has been reported increased UPR activity in the affected brain regions of Parkinson's patients ⁽¹⁴⁵⁾. Also, increased PERK and eIF2 α levels in hippocampus neurons of Alzheimer's disease have been found ⁽¹⁴⁶⁾. Protein folding and mis-folding in the ER as well as their accumulation in ER and mitochondria may play a key role in the development of neurodegenerative diseases. In turn, the impairment of ER-mitochondrial interplay is a common mechanism of different neurodegenerative diseases. In this respect, recent findings show a link between protein conformational anomalies and metabolic dysfunction, and the role of MAM and ER-mitochondria crosstalk in neurodegeneration. ⁽¹⁴⁷⁾.

Among neuropathology affecting mitochondria function, Leber's hereditary optic neuropathy (LHON) was first described by the German ophthalmologist Theodore Leber (1840-1917) ⁽¹⁴⁸⁾. It is a maternally inherited disease, transmitted through mitochondrial DNA ⁽¹⁴⁹⁾, that is characterized by acute or subacute painless central visual loss in otherwise healthy persons ⁽¹⁵⁰⁾. Moreover, LHON was the first clinically characterized mitochondrial disorder and remains the most commonly recognized mitochondrial disorder worldwide. At the molecular level, it results from mutations in the mitochondrial DNA ⁽¹⁵¹⁾. All of which are missense mutations, and were found to affect the nicotinamide adenine dinucleotide dehydrogenase (NADH dehydrogenase) subunit genes encoding proteins involved in Complex 1 of the respiratory chain, thereby interrupting oxidative phosphorylation ⁽¹⁵²⁾.

We have collaborated with the laboratory of Biochemistry of the Centre Hospitalo-Universitaire d'Angers in order to analyze the profile of ER stress response of fibroblasts from healthy subjects and those from patients displaying LOHN disease. Our contribution in this study is limited since the major proportion of the manuscript presented as annex of my Thesis was performed in the laboratory of Biochemistry.

Our results show that LHON patients exhibit enhanced ER stress response under basal conditions. Interestingly, treatment with Tudca, the ER stress inhibitor, prevented the overexpression of the ER stress markers. These data highlight the involvement of ER stress in LHON disease. However, it remains to be determined whether ER stress is the cause or the consequence of LHON disease.

THE METABOLOMIC SIGNATURE OF LEBER HEREDITARY OPTIC NEUROPATHY REVEALS ENDOPLASMIC RETICULUM STRESS

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Running title: ER stress in LHON

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Abbreviations: **CHOP:** CCAAT/enhancer-binding protein (C/EBP) homologous protein; **eIF2 α :** eukaryotic initiation factor 2 α ; **ER:** Endoplasmic Reticulum; **ICR:** Integrated Cell Response; **LHON:** Leber's Hereditary Optic Neuropathy; **mtDNA:** mitochondrial DNA; **ND:** NADH dehydrogenase; **OPLS-DA:** Orthogonal Partial Least Squares – Discriminant Analysis; **OXPHOS:** Oxidative Phosphorylation; **PC:** Phosphatidylcholine; **PCA:** Principal Component Analysis; **PERK:** Protein kinase RNA-activated (PKR)-like ER kinase; **RGC:** Retinal Ganglion Cells. **SM:** Sphingomyelin; **TCA cycle:** Tricarboxylic acid cycle; **TUDCA:** Tauroursodeoxycholic acid; **UPR:** Unfolded Protein Response; **VIP:** Variable Importance in the Projection.

ABSTRACT

Leber's hereditary optic neuropathy (LHON, MIM#535000), the commonest mitochondrial DNA-related disease, is caused by mutations affecting mitochondrial complex I. The clinical expression of the disorder, usually occurring in young adults, is typically characterized by subacute, usually sequential, bilateral visual loss, resulting from the degeneration of retinal ganglion cells. Since the precise action of mitochondrial DNA mutations on the overall cell metabolism in LHON is unknown, we investigated the metabolomic profile of the disease. High performance liquid chromatography coupled with tandem mass spectrometry was used to quantify 188 metabolites in fibroblasts from 16 LHON patients and 8 healthy controls. Latent variable-based statistical methods were used to identify discriminating metabolites. One hundred and twenty-four of the metabolites were considered to be accurately quantified. A supervised orthogonal partial least squares discriminant analysis model separating LHON patients from controls showed good predictive capability ($Q^2_{\text{cumulated}}=0.57$). Thirty-eight metabolites appeared to be the most significant variables, defining a LHON metabolic signature that revealed decreased concentrations of all proteinogenic amino acids, spermidine, putrescine, isovaleryl-carnitine, propionyl-carnitine and five sphingomyelin species, together with increased concentrations of 10 phosphatidylcholine species. This signature was not reproduced by the inhibition of complex I with rotenone or piericidin A in control fibroblasts nor was it found in the plasma from LHON patients. The importance of sphingomyelins and phosphatidylcholines in the LHON signature, together with the decreased amino acid pool, suggested an involvement of the endoplasmic reticulum (ER). This was confirmed by the significantly increased phosphorylation of PERK and eIF2 α , as well as the greater expression of CHOP, in LHON fibroblasts, all these changes being reversed by the ER stress inhibitor, TUDCA. Thus, our metabolomic analysis reveals a pharmacologically-reversible ER stress in complex I-related LHON fibroblasts, a finding that may open up new therapeutic perspectives for the treatment of LHON with ER-targeting drugs.

INTRODUCTION

Leber's hereditary optic neuropathy (LHON, MIM#535000) was first described in 1871 by the German ophthalmologist Theodore Leber (Leber, 1871). LHON, with a prevalence estimated at 1/31 000 to 1/50 000 in Europe, is the commonest inherited mitochondrial disorder due to mitochondrial DNA (mtDNA) mutations (Man *et al.*, 2003; Puomila *et al.*, 2007; Masciallino *et al.*, 2012). The disease is clinically characterized by a subacute, severe and painless, sequential, bilateral loss of vision occurring within weeks or months of onset (Newman *et al.*, 2005; Milea *et al.*, 2010; La Morgia *et al.*, 2014; Yu-Wai-Man *et al.*, 2009; Yu-Wai-Man *et al.*, 2011). Fundoscopic examination during the acute phase shows telangiectatic microangiopathy and swelling of the retinal nerve fibre layer around the optic disc (Leruez *et al.*, 2014). As the disease progresses toward the chronic phase, atrophy of the retinal ganglion cells (RGCs) is evidenced by the manifestation of optic disc pallor. Typically, the visual field presents a central or centrocecal visual defect together with a relatively preserved peripheral vision. In most cases, LHON affects young adults, but loss of vision may occur at ages varying from childhood to 87 years (Giraudet *et al.*, 2011). LHON typically affects males more frequently than females, with a predominance of 80-90% (Newman *et al.*, 2005; Fraser *et al.*, 2010). In rare cases, the visual loss may be associated with additional non-visual clinical features, such as abnormalities of cardiac conduction, skeletal muscle alterations, dystonia, spasticity, psychiatric disturbances, encephalopathy, multiple-sclerosis-like and Leigh-like phenotypes (Harding *et al.*, 1992; Jun *et al.*, 1994; Funalot *et al.*, 2002; Pfeffer *et al.*, 2013).

The genetic cause of the disease was first evidenced in 1988 by Douglas Cecil Wallace and his team (Wallace *et al.*, 1988). The identification of the m.11778G>A mtDNA mutation in the *MT-ND4* gene was followed by the discovery of the m.3460G>A and m.14484T>C

mtDNA mutations, respectively in the *MT-ND1* (subunit 1 of the mitochondrial NADH dehydrogenase or complex I of the respiratory chain) and *MT-ND6* genes (Howel *et al.*, 1991; Huoponen *et al.*, 1991; Mackey *et al.*, 1992; Johns *et al.*, 1992). These three mtDNA pathogenic variants account for about 95% of all LHON cases in Europe. The m.11778G>A mutation is the most common, whereas the m.3460G>A mutation is usually considered the most severe, and the m.14484T>C mutation has the best visual prognosis (Mackey *et al.*, 1992). A dozen other rare mtDNA pathogenic variants, affecting mainly the ND genes, account for the remaining 5% of LHON cases (<http://www.mitomap.org/MITOMAP>, Lott *et al.*, 2013; Achilli *et al.*, 2012). LHON has incomplete and variable penetrance (Howel and Mackey 1998), estimated at about 50% in males and 10% in females (Yu-Wai-Man *et al.*, 2011). The mtDNA mutation rate, environmental and hormonal factors (Yu-Wai-Man *et al.*, 2009; Giordano *et al.*, 2011; Giordano *et al.*, 2015), mitochondrial (Hudson *et al.*, 2007) and nuclear (Bu and Rotter, 1991; Nakamura *et al.*, 1993; Hudson *et al.*, 2005; Sankar *et al.*, 2008; Jiang *et al.*, 2015) genetic backgrounds, as well as individual ability to increase mitochondrial biogenesis (Giordano *et al.*, 2014), have all been reported to influence LHON penetrance.

The pathophysiology of LHON remains to be elucidated. The pathogenic mechanism underlying the disease is currently believed to be linked to vulnerability to an energetic defect of the unmyelinated prelaminar RGC axons (Carelli *et al.*, 2004). Our earlier work has shown that LHON fibroblasts carrying the three main mtDNA mutations, i.e. m.11778G>A, m.3460G>A and m.14484T>C, are characterized by a significant decrease of complex I enzymatic activity, a lower respiratory reserve capacity, a lower complex I-driven respiratory rate leading to decreased ATP synthesis and partial OXPHOS uncoupling with reduced mitochondrial membrane potential, with no morphological alteration of the mitochondrial network (Chevrollier *et al.*, 2008; Angebault *et al.*, 2011). A similar defect of ATP synthesis

was also reported in transmitochondrial cybrids carrying the three main LHON mutations (Baracca *et al.*, 2005), associated with the disruption of glutamate transport (Beretta *et al.*, 2004) and increased production of reactive oxygen species (Wong *et al.*, 2002). The ATP defect was also observed *in vivo* by ^{31}P -MRS in the skeletal muscle of patients carrying the three main LHON mutations (Lodi *et al.*, 1997).

A metabolomic strategy, based on recent technological advances, now allows the accurate identification and quantification of small biological molecules, opening up the possibility of assessing the metabolic consequences of mitochondrial dysfunction. Indeed, most energy-dependent cell functions and metabolisms are affected by impaired OXPHOS. We have performed a comparative targeted metabolomic study of the well-characterized, homogeneous mitochondrial LHON disorder involving moderate complex I deficiency. This approach led us to identify a specific metabolic signature of LHON revealing an endoplasmic reticulum stress.

MATERIALS AND METHODS

Patients

Written informed consent was obtained from all patients participating in the study, which was approved by the Ethical Committee of the University Hospital of Angers (*Comité de Protection des Personnes CPP Ouest II – Angers, France*; Identification number: CPP CB 2014/02; Declaration number: DC-2011-1467 and Authorization number: AC-2012-1507). We studied primary skin fibroblasts from 16 individuals each carrying one mutation associated with LHON (m.11778G>A, m.3460G>A and m.14484T>C; rCRS/NC_012920.1), and from 8 healthy subjects, hereafter referred to as controls. Fasting blood samples, immediately centrifuged for plasma conservation at -80°C , obtained from a subset of 6 LHON patients were also examined.

Cell cultures

Fibroblasts were cultured in a medium consisting of 2/3 Dulbecco's modified Eagle medium (DMEM-F12, Jacques Boy Institute of Biotechnology, Reims, France), 1/3 Amniomax (Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (PAA, Pasching, Austria) at 37°C, 5% CO₂. In order to avoid artefacts due to senescence, all the experiments were conducted on fibroblast cultures between the 6th and the 25th passages (Hutter *et al.*, 2004).

Complex I enzymatic activity and inhibition

Complex I activity was measured on cell lysates. Cells were disrupted by freezing in liquid nitrogen followed by rapid thawing at 37°C. Lysates were then enriched in mitochondria by centrifugation (16,000 g, 1 min at 4°C), washed once in cell buffer (250 µl/10⁶ cells) and sonicated 6 x 5 seconds on ice with a Branson Sonic Power sonicator (SmithKline Company, Danbury, CT, USA). NADH ubiquinone reductase (NUR) activity was measured as described elsewhere (Angebault *et al.*, 2011) at 37°C on a UVmc2 spectrophotometer (SAFAS, Monaco). NADH (0.10 mM) was added to initiate the reaction. Rotenone (5 µM) was used to determine the background rate. To investigate the metabolomic effect of experimental partial inhibition of complex I, healthy fibroblasts were treated for 48 h by either the vehicle (ethanol 1/2500) or 1 µM of rotenone or 5 nM of piericidin A before measuring complex I enzymatic activities. These inhibitor concentrations, aimed at decreasing complex I activity by 20 to 60%, were determined using a calibration range. Complex I activity was normalized with respect to citrate synthase activity that was assayed by a standard procedure (Bonnet *et al.*, 2008).

Extraction of metabolites

When 70% confluence was obtained, the culture medium was removed and fibroblasts were harvested by trypsination and washed twice in a cold, phosphate-buffered saline 1X (PBS-1X) solution and 10% fetal bovine serum (PAA, Pasching, Austria). After centrifugation (300 g for 5 minutes at 4°C), the supernatant was eliminated and the cell pellet was dissolved in 100 µL of a cold PBS-1X solution. Fifty microliters were used for protein dosage, the remaining 50 µL (~ 2.10⁶ cells) being used for the extraction of metabolites. The metabolic quenching was completed by adding 100 µL of a cold ethanol/PBS-1X (85:15, v/v) solution to the cell pellet obtained after a second run of centrifugation (300 g for 5 minutes at 4°C) and elimination of the supernatant. After vortexing, the mixture was transferred to a 0.5 ml homogenizer tube prefilled with ceramic beads. Cell lysis and protein extraction was achieved in a Precellys®24 homogenizer (Bertin Corp., Rockville, MD, USA) by two cycles of grinding (40 seconds at 6500 rpm, followed by 30 seconds at 6000 rpm) at 4°C. The resulting homogenate was centrifuged at 20 000 g for 10 min at 4°C and the supernatant was stored at -80°C until analysis.

Metabolomic analysis of fibroblast homogenates

We applied a targeted, quantitative metabolomic approach to the cell extracts using the Biocrates AbsoluteIDQ p180 kit (Biocrates Life sciences AG, Innsbruck, Austria). This kit, in combination with a AB Sciex QTRAP 5500 (Life Sciences SCIEX, Villebon sur Yvette, France) mass spectrometer, enables quantification of up to 188 different endogenous molecules, including acylcarnitines (40), amino acids (21), biogenic amines (21), glycerophospholipids (90), sphingolipids (15), and sugar (1). Flow-injection analysis (FIA-MS/MS) was used for quantifying acylcarnitines, glycerophospholipids, sphingolipids and sugar, whereas liquid chromatography (LC) allowed the separation of amino acids and

biogenic amines prior to detection with mass spectrometry (LC-MS/MS). Samples were prepared according to the Biocrates Kit User Manual. Briefly, after thawing on ice, 10 μ L of each sample (cell-lysate homogenate supernatants) were added to the center of the filter placed on the upper wall of the well in a 96-well plate. Metabolites were extracted in a methanol solution using ammonium acetate after drying the filter spot under nitrogen flow and derivatizing with phenylisothiocyanate for the quantification of amino acids and biogenic amines. The extracts were finally diluted with MS running solvent before FIA and LC-MS/MS analysis. After validation of the three levels of quality controls (QCs) used in the kit, the metabolite concentrations were normalized with respect to protein cell extract concentrations.

Amino acid chromatography and acylcarnitine dosages in plasma from LHON patients

Forty-nine amino acids and amino acid-derived molecules were quantified using an API 3000 Triple Quadrupole Mass Spectrometer AB Sciex® (Life Sciences SCIEX, Villebon sur Yvette, France) on 6 plasma samples from patients symptomatic of LHON. Free carnitine (CN) and acylcarnitines (AcylCN) in the same plasma samples were quantified in butyl-ester forms using isotope-dilution, electrospray-ionization tandem mass spectrometry (ESI-MS/MS) method (Mingorance *et al*, 2012).

Assessment of endoplasmic reticulum stress

Subsets of three LHON fibroblasts (P5 carrying the m.14484T>C mutation, and P10 and P11 carrying the m.11778G>A mutation), and three control fibroblasts (C3, C7, and C8) were used for the investigation of endoplasmic reticulum (ER) stress. Cells were homogenized and lysed. Proteins (40 μ g) were separated on 4-12% NuPage gels (ThermoFisher Scientific,

Rockford, IL, USA). Blots were probed with anti-PERK, phospho-PERK, eIF2 α , phospho-eIF2 α , CHOP (Cell Signaling, Danvers, MA, USA) overnight at 4°C. A polyclonal mouse anti-human β -actin antibody (1/2000e) (Sigma-Aldrich, Saint Quentin Fallavier, France) was used for standardizing the protein gel loading. The membranes were then washed at least three times in a Tris-buffer solution containing 0.05% Tween 20, and incubated for 1 hour at room temperature with the appropriate secondary antibody (anti-rabbit or polyclonal mouse antibody). The protein-antibody complexes were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific), in an LAS4000 revelation machine. The ER stress inhibitor TUDCA (100 μ M) was used to treat LHON and control fibroblasts during 24 hours before Western blotting.

Statistical analysis

Before performing statistical analysis, raw data were examined in order to eliminate the metabolites that were not accurately measured, i.e. metabolites with concentrations below the lower limit of quantitation (LLOQ) or above the upper limit of quantitation (ULOQ). When all the concentrations for a given metabolite were not between the LLOQ and the ULOQ values, we used a Chi-square test to assess whether the proportion of concentration values out of range in LHON and control samples was similar, that is, whether there was a LHON effect with an out-of-range concentration. The significance level for this test was set at $p \leq 0.1$. Here, we were more interested in detecting metabolites that were important for class discrimination than in their concentration *per se*. When the independence hypothesis could not be rejected and more than 30% of the concentration values were not considered accurately measured, the metabolite was not used for statistical analysis, otherwise it was retained. The bilateral Student's t-test or the Mann-Whitney test was used for group comparisons involving quantitative variables. Differences were considered statistically significant at $p \leq 0.05$, unless

otherwise mentioned. Univariate analyses were conducted using R software, version 3.1.1 (The R Foundation for Statistical Computing, Vienna, Austria).

Multivariate analysis was performed on unit variance-scaled data. Principal component analysis (PCA) was employed to detect similar samples forming clusters and outliers. Orthogonal partial least squares discriminant analysis (OPLS-DA), a supervised method of pattern recognition, was further used to maximize the variations between groups and to determine the contributing variables. The quality of models was validated by determining two parameters: R^2 (goodness of fit) and $Q^2_{\text{cumulated}}$ (goodness of prediction). In the model with the best predictive capabilities, variables were selected on the basis of the variable importance in the projection (VIP) and coefficient values. VIP values summarize the importance of each variable for the OPLS-DA model, whereas coefficient values summarize the relationship between the realizations of the Y variable (LHON or control) and the X variables (matrix of measured metabolites). Variables with a VIP value greater than 1 are considered important for group discrimination in predictive models. Regression coefficient values are very similar to coefficients obtained from multiple linear regressions. Plotting VIP *versus* coefficient values – a procedure called the “volcano” plot – allows the selection of important variables in PLS models. Multivariate data analysis was conducted using the SIMCA-P software 13.0 (Umetrics, Umeå, Sweden).

RESULTS

Population

Table 1 gives details of the cohort of patients and controls participating in the study.

Impaired complex I enzymatic activity in LHON and rotenone- or piericidin A-treated fibroblasts

Compared to controls, the average complex I enzymatic activity in LHON fibroblasts was reduced by 33% ($p < 0.001$) (Figure 1A), whereas complex I inhibition was 50% ($p < 0.05$) in rotenone-treated fibroblasts, and 57% ($p < 0.01$) in piericidin A-treated cells (Figure 1B).

Metabolomic signature of LHON fibroblasts

Extracts from fibroblasts of 16 LHON mutation carriers and 8 controls, and from fibroblasts treated by either rotenone or piericidin A, were analyzed in two separate runs. Of the 188 metabolites analyzed, 123 were retained for statistical analysis after quality control and dynamic range validation, viz. 5/40 acylcarnitines, 19/21 amino acids, 7/21 biogenic amines 78/90 lyso- and glycerophosphatidylcholines, 13/15 sphingomyelins and 1 hexose. The raw data are shown in Supplementary Table 1. In extracts from control fibroblasts treated with rotenone ($n=9$) or piericidin A ($n=10$) and from untreated control fibroblasts ($n=10$), 130 metabolites were considered accurately measured and retained for statistical analysis, viz. 5/40 acylcarnitines, 21/21 amino acids, 9/21 biogenic amines, 82/90 glycerophosphatidylcholines, 12/15 sphingomyelins and 1 hexose. The raw data are shown in Supplementary Table 2. Principal component analysis of normalized data showed a clear separation between LHON and control fibroblasts, with no outliers. Supervised OPLS-DA showed an even better separation between the two groups, with good predictive capabilities ($Q^2_{\text{cumulated}} = 0.57$) (Figure 2A).

Figure 3 shows the main metabolites discriminating between LHON fibroblasts and controls with high VIP and coefficient values. Strikingly, the concentration of all the proteinogenic

amino acids measured was lower in LHON fibroblasts than in controls. Similarly, the concentrations of two polyamines, spermidine and its precursor putrescine, along with those of acylcarnitines C3 and C5, and 6 sphingomyelins, were also lower in LHON fibroblasts than in controls. Conversely, the concentration of 10 phosphatidylcholines was higher in LHON fibroblasts than in controls.

Metabolomic signature of rotenone- and piericidin A-treated fibroblasts

The OPLS-DA model comparing vehicle-treated fibroblasts with those treated with the two complex I inhibitors, rotenone and piericidin A, showed a clear distinction in each case (Figure 2B), (Raw data shown in Supplementary Table 2). The predictive capabilities of the two models were excellent, with high values of $Q^2_{\text{cumulated}}$ (0.98 and 0.89, respectively).

In both cases, thirty-two metabolites were the best discriminators between controls and CxI-inhibited fibroblasts (Figures 4 & 5).

Interestingly, the metabolic signatures of rotenone and piericidin A overlap greatly, sharing 20 of the 32 most discriminating metabolites (Table 2). However, only five of these 20 metabolites have also been identified as discriminants in LHON fibroblasts, i.e. isovaleryl-carnitine (C5), aspartate, asparagine, glycine, and PC aa 36:6.

Amino acids and acylcarnitines are not altered in the plasma from LHON patients

The routine analysis of plasma samples from six LHON patients revealed no abnormality in the concentration of amino acids and acylcarnitines (data not shown).

LHON fibroblasts manifest endoplasmic reticulum stress

Since sphingomyelins and phosphatidylcholines are synthesized in the endoplasmic reticulum (ER), and ER stress is associated with altered protein synthesis, the LHON metabolic signature pointed to a possible involvement of ER stress. In order to investigate whether ER stress is present in LHON disease, Western blots for the components of the unfolded protein response (UPR) of the ER stress were performed on protein extracts from three LHON fibroblasts and three controls. As shown in Figure 6, increased phosphorylation of PERK (+344%) and eIF2 α (+265%), as well as in the expression of the CHOP protein (+248%) was observed in LHON cells compared to controls ($p < 0.05$). The increased phosphorylation of eIF2 α observed in LHON fibroblasts was significantly reduced by 82% when the cells were cultured for 24 hours with TUDCA, an ER stress inhibitor ($p < 0.05$). These findings indicate that the ER stress response is activated in LHON.

DISCUSSION

To our knowledge, this is the first report of a combined metabolomic and lipidomic signature associated with LHON, the commonest inherited optic neuropathy and the most frequent disease related to an mtDNA disorder. Our results, based on the comparison of 16 fibroblast cell lines, each carrying one of the three primary LHON mutations, and 8 control fibroblast cell lines, revealed a statistically significant LHON profile composed of 28 under-represented metabolites belonging to two groups of molecules: amino acids and sphingomyelins, and 10 over-represented metabolites, all of which were phosphatidylcholines. Further statistical analyses comparing the 16 LHON fibroblast cell lines with respect to the gender of the LHON patients (10 males vs. 6 females), clinical status (12 affected vs. 4 unaffected), mutation (11 cases with the m.11778 G>A mutation vs. five cases with the m.3640 G>A or the m.14484 T>C mutations), and age (8 patients aged under 40 years vs. 8 patients aged over 40 years)

revealed no significant differences, suggesting that the metabolomic signature inferred from the 16 LHON fibroblast cell lines studied may be considered to define a latent LHON background, common to all individuals bearing one of the three pathogenic mutations.

This metabolomic signature is characterized by the decrease of all proteinogenic amino acids, associated with two acylcarnitines (C3 and C5) and two biogenic amines, putrescine and spermidine, which are signaling molecules derived from amino acids. The link between the metabolism of amino acids and mitochondrial bioenergetics has long been established since the tricarboxylic acid cycle is known to be involved in the biosynthesis and catabolism of amino acids. Other metabolic analyses have revealed the decreased concentration of some amino acids in animal models of complex I deficiency, for example in the *Ndufs4*^{-/-} mouse mimicking the Leigh syndrome (Johnson *et al.*, 2013) and in the *Ndufs2* mutated *C. elegans* worms (Morgan *et al.*, 2015), whereas the concentration of other amino acids simultaneously increased in these models. These findings suggest that the alteration of complex I affects the metabolism of amino acids, although to the best of our knowledge, no generalized reduction of all proteinogenic amino acids has ever been reported so far, either in LHON or in any other respiratory chain disorder. The presence of isovaleryl-carnitine (C5) and propionyl-carnitine (C3) in this metabolomic signature concords with the modification of the amino acid profile, since these carnitines are intermediate products of amino acid catabolism. In fact, a study on the *Ndufs4*^{-/-} mouse has also reported decreased concentrations of C3 in the blood and C5 in the heart (Leong *et al.*, 2012).

In addition, the metabolomics signature of LHON revealed five under-represented sphingomyelins and 10 over-represented phosphatidylcholines, which represent two metabolically related families of phospholipids since phosphatidylcholines are precursors of

sphingomyelin. Indeed, the synthesis of sphingomyelin starts in the endoplasmic reticulum (ER) with the condensation of a palmitic acid to serine, a reaction catalyzed by serine-palmitoyl transferase. Serine being one of the most significantly reduced metabolites in the LHON signature, the lack of serine could impair the synthesis of sphingomyelin, leading to the accumulation of phosphatidylcholines, in agreement with the signature we found. Further analyses of 10 of the 69 phosphatidylcholines, for which the LHON signature showed increased concentrations, indicated that most of these were poly-unsaturated acyl-alkyl-phosphatidylcholines (ae) that may offer protection against oxidative stress (Engelmann, 2004).

We wondered how the LHON mutations might have led to the metabolomic signature observed. The reduction of all the amino acids and some sphingomyelins, together with the increase of some phosphatidylcholines, were reminiscent of an ER dysfunction, the ER being the central subcellular compartment for protein and lipid syntheses. Our findings prompted us to assess the specific markers of the unfolded protein response (UPR) that may have caused ER stress in LHON fibroblasts. This study revealed an induction of PERK and eIF2 α phosphorylation, together with increased amounts of CHOP, suggestive of latent ER stress, which we showed to be attenuated by TUDCA. In concordance with our findings, phosphatidylcholine synthesis has been reported to increase in glioma cells after the induction of ER stress (Epple *et al.*, 2013), whereas the synthesis of sphingolipids was repressed under the same conditions in yeast (Liu *et al.*, 2012). Interestingly, the neurotoxic complex I inhibitor, MPP⁺ (1-methyl-4-phenylpyridinium), was also recently reported to induce ER stress and increase the concentration of six phosphatidylcholines in dopaminergic neurons (Krug *et al.*, 2014). The concentration of only four amino acids was found decreased in this model despite the considerably altered genes involved in amino acid metabolism that were

identified by transcriptomic analysis. Nevertheless, a global decrease of the amino acid pool has never been reported in the context of an ER stress. This process may be related to the integrated stress response (ISR), which is a globally adaptive response to many forms of cell stress (Jiang and Wek, 2005). ISR regulates amino acid metabolism by eIF2 α phosphorylation, which attenuates protein synthesis, increases the expression of genes involved in amino acid import and biosynthesis, and modulates the ubiquitin-proteasome pathway, an ATP-dependent process (Suraweera *et al.*, 2012; Harding *et al.*, 2003). Thus, the reduced ATP production we previously reported in LHON fibroblasts (Chevrollier *et al.*, 2008) may inhibit the proteasome, thereby leading to the reduction of the amino acid pool and to the induction of ER stress (Suraweera *et al.*, 2012), with the consequences observed downstream on sphingomyelin and phosphatidylcholine syntheses. This concept would satisfactorily embrace all the results we have presented here.

We hypothesized that reduced NADH dehydrogenase activity might be the key process underlying the pathophysiological mechanism of ER stress. To test this hypothesis, we investigated the consequences of this reduced enzyme activity on the metabolomic signature of two groups of wild-type fibroblasts treated with drugs inhibiting complex I activity, one group being incubated with rotenone, an isoflavonoid, and the other with piericidin A, a Co-Q antagonist. Statistical models distinguishing control fibroblasts from rotenone or piericidin A-treated fibroblasts with excellent predictive capabilities were found. In both models, 32 metabolites were the most significant features for differentiating the groups. Nineteen of these were amino acids, phosphatidylcholines, sphingomyelins, putrescine and acylcarnitines, shared by the two groups of the wild-type fibroblasts, whereas the others were lysophosphatidylcholines, phosphatidylcholines, and acylcarnitines, specific to each group. Surprisingly, neither of the metabolomic signatures of the two groups of wild-type fibroblasts

reproduced the signature of LHON fibroblasts. In fact, these signatures revealed that only a few amino acids were reduced, whereas others, such as alanine, were increased; some phosphatidylcholines were reduced, and new metabolites, such as lysophosphatidylcholines, had differentiating profiles. Nevertheless, the wild-type fibroblasts, treated with drugs inhibiting complex I activity, shared a few metabolites with LHON fibroblasts, such as aspartic acid, asparagine, glycine and isovaleryl-carnitine (C5), and a single phosphatidylcholine. The divergence between the signatures of fibroblasts treated with inhibitory drugs and LHON fibroblasts probably reflects the different effects on complex I activity of the two drugs tested, i.e. rotenone and piericidin A, on one hand, and the mtDNA mutations in the *ND1*, *ND4* and *ND6* genes, on the other, although all of these sharply inhibit complex I activity.

In conclusion, our targeted metabolomic approach has revealed an unexpected LHON metabolomic/lipidomic signature with globally decreased amino acid concentrations, and a novel pathophysiological mechanism involving ER dysfunction that, taken together, may be linked to the integrated stress response paradigm. These findings are concordant with the results of other “omics” studies performed on cells from LHON patients. Thus, a recent proteomics study has reported downregulation of the quality control pathways in LHON fibroblasts carrying the m.11778G>A mutation (Tun *et al.*, 2014); and a transcriptomic approach highlighted UPR activation and altered protein synthesis as the main features of mitochondrial respiratory chain disorders such as LHON (Cortopassi *et al.*, 2006). Although currently restricted to cell samples since no significant signature has yet been found in the plasma from LHON patients, these novel biomarkers should eventually prove useful for the assessment and diagnosis of the disease. Nevertheless, the involvement of ER stress in

LHON, and possibly in the pathophysiology of RGCs, opens up new perspectives for the investigation and treatment of the disease by the use of ER-targeting drugs.

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For Peer Review

TABLES AND FIGURES LEGENDS

Table 1: Description of the cohort of patients and controls participating in the study.

Table 2: The most discriminating metabolites between fibroblasts treated with rotenone or piericidin A and control fibroblasts. PC aa: diacyl phosphatidylcholine; PC ae: acyl-alkyl phosphatidylcholine; SM: sphingomyelin; SM(OH): hydroxysphingomyelin; Lyso PC a: acyl-lysophosphatidylcholine; t4-OH-Pro: trans-4-hydroxyproline; and H1: sum of hexoses. In bold type, the metabolites that were also identified as discriminants in LHON fibroblasts.

Figure 1: Enzymatic activity of complex I. **A:** In fibroblasts carrying a LHON mutation and control fibroblasts; and **B:** In fibroblasts treated with rotenone or piericidin A, and control fibroblasts. Results were normalized with respect to citrate synthase (CS) activity (CI/CS). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2: LHON PCA and OPLS-DA models and OPLS-DA models for rotenone and piericidin A-treated fibroblasts. **A:** PCA (left) and OPLS-DA (right) of metabolomic data from control fibroblasts ($n=8$, blue dots) and fibroblasts carrying a LHON mutation ($n=16$, green dots). With PCA, the first two principal components (PC1 and PC2) explain more than 75% of the total variance. There are no outliers, and the second component (PC2) clearly separates the two groups of fibroblasts. With OPLS-DA, the first latent variable (LV1) shows even clearer separation between the two groups of fibroblasts than the second component in PCA. The supervised model has good predictive discriminant properties ($Q^2_{\text{cumulated}} = 0.57$) and explains up to 68% of the variance of the Y vector. **B:** OPLS-DA models for fibroblasts treated with rotenone (left) and piericidin A (right) versus control fibroblasts. The metabolomic effects of rotenone and piericidin A on control fibroblasts led to the construction of two models that clearly distinguished between treated fibroblasts (blue dots) and control fibroblasts (green dots), with good predictive capabilities reflected by high values of $Q^2_{\text{cumulated}}$ (0.98 and 0.89, respectively).

Figure 3: Coefficient versus VIP or “volcano” plot for the OPLS-DA model obtained from the metabolomics analysis of LHON fibroblasts and controls. Only the most discriminating metabolites with high VIP values (>1) and coefficient values (≥ 0.02 or $\leq -$

0.02) have been labeled. Negative coefficients (left) indicate lower metabolite concentrations in LHON fibroblasts than in controls whereas positive coefficients (right) indicate metabolites with higher concentrations in LHON fibroblasts than in controls. The metabolic signature of LHON is characterized by higher levels of 10 phosphatidylcholines (PC, light blue bubbles) and lower levels of 19 amino acids (green bubbles), 2 acylcarnitines (brown bubbles), 5 sphingomyelins (yellow bubbles), 2 biogenic amines (pink bubbles). VIP: variable importance in the projection; PC aa: diacyl phosphatidylcholine; PC ae: acyl-alkyl phosphatidylcholine; SM: sphingomyelin; SM(OH): hydroxysphingomyelin; C3: propionylcarnitine; and C5: isovalerylcarnitine.

Figure 4: Coefficient versus VIP or the «volcano» plot for the OPLS-DA model obtained from the metabolomic analysis of fibroblasts treated with rotenone. Only the most discriminating metabolites having high VIP values (>1) and coefficient values (≥ 0.02 or ≤ -0.02) have been labeled. Negative coefficients (left) indicate diminished metabolite concentrations in rotenone-treated fibroblasts versus control fibroblasts, whereas positive coefficients (right) indicate increased metabolite concentrations. The metabolomic signature of rotenone is accompanied by increased levels of 1 acyl-lysophosphatidylcholine (lysoPC a, dark blue bubble), 6 phosphatidylcholines (PC, blue bubbles), 3 acylcarnitines (brown bubbles), 1 biogenic amine (pink bubble), and 1 amino acid (green bubble), and decreased levels in 9 phosphatidylcholines, 7 amino acids, 2 sphingomyelins (yellow bubbles), 1 acyl carnitine and 1 hexose (dark yellow bubble). Red-rimmed bubbles indicate metabolites that are also important in discriminating between fibroblasts treated with piericidin A and control fibroblasts. VIP: variable importance in the projection; PC aa: diacyl phosphatidylcholine; PC ae: acyl-alkyl phosphatidylcholine; SM: sphingomyelin; SM(OH): hydroxysphingomyelin; C2: acetylcarnitine; C5: isovalerylcarnitine; C16: hexadecanoylcarnitine; C18: octadecanoylcarnitine; and H1: sum of hexoses.

Figure 5: Coefficient vs. VIP or the “volcano” plot for the OPLS-DA model derived from the metabolomic analysis of fibroblasts treated with piericidin A. Only the most discriminating metabolites with high VIP (>1) and coefficient values (≥ 0.02 or ≤ -0.02) have been labeled. Negative coefficients (left) indicate diminished metabolite concentrations in piericidine A-treated versus control fibroblasts, whereas positive coefficients (right) indicate increased metabolite concentrations. The metabolic signature of piericidin A is similar to that

of rotenone, and is accompanied by increased levels of 3 lysophosphatidylcholines (lysoPC, dark blue bubbles), 3 phosphatidylcholines (PC, blue bubbles), 3 acylcarnitines (brown bubbles), 2 biogenic amines (pink bubbles) and 1 amino acid (green bubble) and decreased levels of 11 phosphatidylcholines, 4 amino acids, 2 sphingomyelins (yellow bubbles) and 1 acyl carnitine. Red-rimmed bubbles indicate metabolites that are also important in discriminating between fibroblasts treated with rotenone-treated and control fibroblasts. VIP: variable importance in the projection; lysoPC a: acyl-lysophosphatidylcholine; PC aa: diacyl phosphatidylcholine; PC ae: acyl-alkyl phosphatidylcholine; SM: sphingomyelin; SM(OH): hydroxysphingomyelin; C2: acetylcarnitine; C5: isovalerylcarnitine; C16: hexadecanoylcarnitine; C18: octadecanoylcarnitine; and t4-OH-Pro: trans-4-hydroxyproline.

Figure 6: Assessment of the PERK-ER stress activating pathway in LHON fibroblasts compared to controls. Western blotting using antibodies against p-PERK, PERK, p-eIF2 α , eIF2 α , CHOP and β -actin (A). Quantification of the ratio of phosphorylation of PERK (B), and eIF2 α (C), and of the ratio of CHOP protein expression expressed as mean \pm SEM in arbitrary units (A.U.) (D). Quantification of the ratio of phosphorylation of eIF2 α in LHON fibroblasts and controls (n=3) treated with the ER stress inhibitor TUDCA (E).

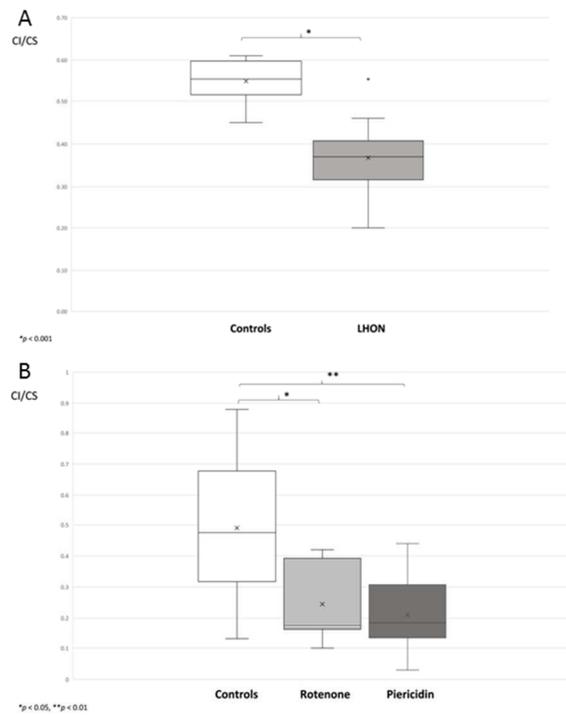
*p < 0.05.

Subjects	Clinical status	Gender	Age	LHON mutation (rate)
P1	Affected	M	21	m.11778G>A (97%)
P2	Affected	M	21	m.14484T>C (100%)
P3	Affected	M	44	m.11778G>A (100%)
P4	Affected	M	36	m.11778G>A (97%)
P5	Affected	M	26	m.14484T>C (100%)
P6	Affected	F	45	m.11778G>A (100%)
P7	Affected	M	54	m.11778G>A (96%)
P9	Affected	M	30	m.11778G>A (100%)
P10	Affected	F	47	m.11778G>A (100%)
P11	Affected	M	27	m.11778G>A (96%)
P12	Affected	M	30	m.3460G>A (100%)
P13	Affected	M	25	m.11778G>A (97%)
P14	Unaffected	F	65	m.11778G>A (100%)
P15	Unaffected	F	68	m.3460G>A (100%)
P16	Unaffected	F	63	m.11778G>A (97%)
P17	Unaffected	F	55	m.3460G>A (100%)
C1		F	25	
C2		H	58	
C3		H	43	
C4		F	21	
C5		H	32	
C6		F	25	
C7		F	47	
C8		F	56	

Table I. Description of the patient cohort.

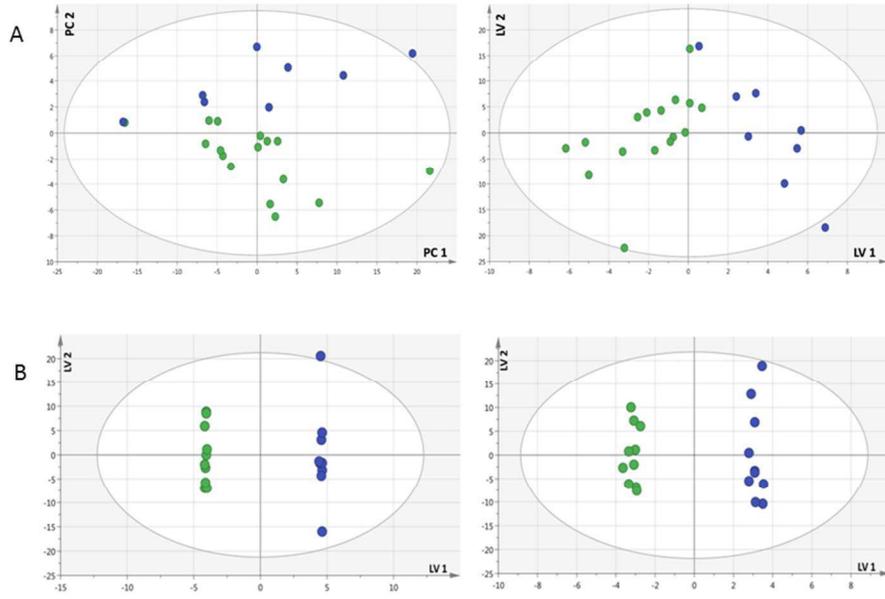
Rotenone	Rotenone and Piericidin A	Piericidin A
↑ Lyso PC 28:0	↑ Acetyl-carnitine (C2)	↑ Lyso PC a16:0, 16:1, 18:1, 18:2
↑ PC aa 30:0, 34:4	↑ Palmitoyl-carnitine (C16)	↑ PC ae 40:2
↑ PC ae 30:0, 30:2	↑ Stearyl-carnitines (C18)	↑ t4-OH-Pro
	↑ PC ae 36:0	
↓ PC aa 42:4	↑ PC ae 36:6	↓ Lyso PC 26:1
↓ SM (OH) 14:1	↑ Putrescine	↓ PC aa 28:1, 42:5, 42:6
↓ Lysine	↑ Alanine	↓ SM 16:0
↓ Arginine		↓ Threonine
↓ Histidine	↓ Isovaleryl-carnitine (C5)	
↓ Ornithine	↓ PC aa 36:4, 38:4, 38:5, 40:6, 42:0	
↓ H1	↓ PC ae 42:1, 42:2, 44:3	
	↓ SM 16:1	
	↓ Aspartic acid	
	↓ Asparagine	
	↓ Glycine	

Table II. Most discriminant metabolites between rotenone and piericidin A treated and control fibroblasts. PC aa: diacyl phosphatidylcholine, PC ae: acyl-alkyl phosphatidylcholine, SM: sphingomyelin, SM(OH): hydroxysphingomyelin, Lyso PC a: acyl-lysophosphatidylcholine, t4-OH-Pro: trans-4-hydroxyproline, H1: sum of hexoses. In bold, the metabolites that were also found discriminant in LHON fibroblasts.



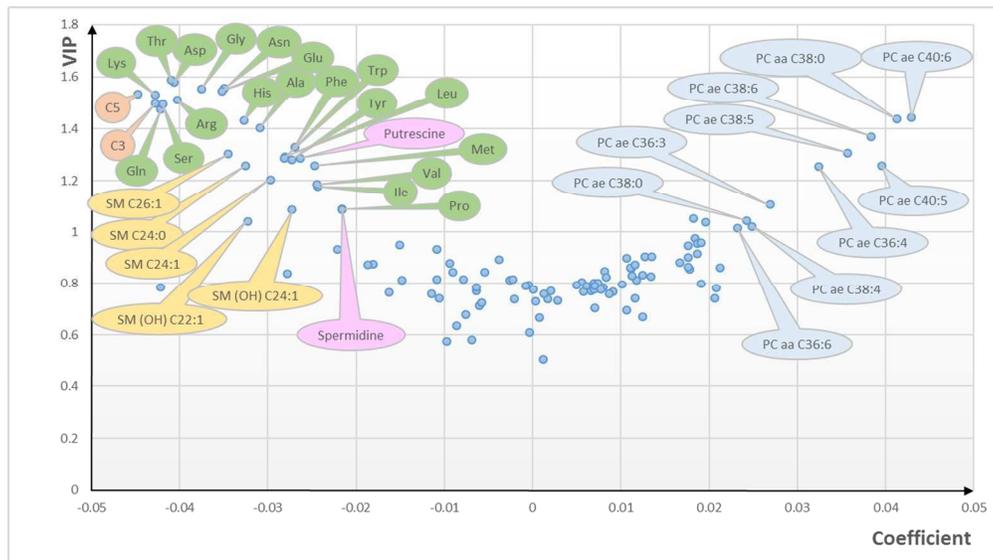
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Review



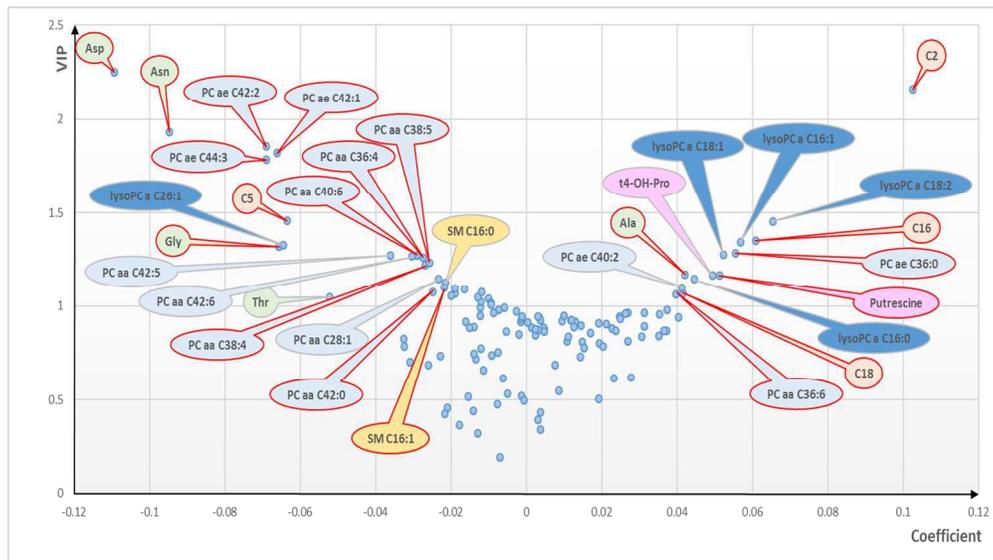
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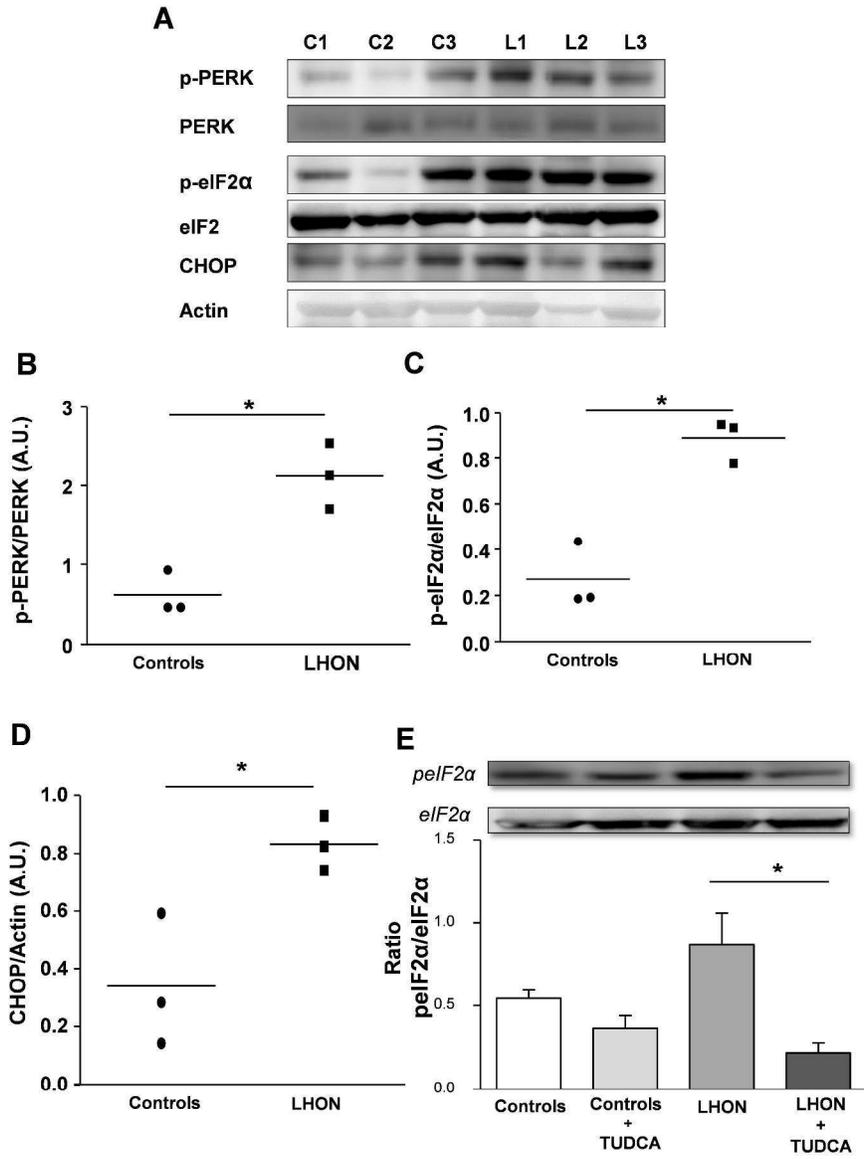
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Peer Review



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Thèse de Doctorat

Zainab SAFIEDEEN

Rôle de l'interaction entre le réticulum endoplasmique et les mitochondries dans la dysfonction endothéliale induite par des microparticules humaines

Role of the interaction between endoplasmic reticulum and mitochondria in endothelial dysfunction induced by human microparticles

Résumé

Le syndrome métabolique est constitué d'une constellation d'anomalies métaboliques telles que l'obésité centrale, une altération de la glycémie à jeun, une hypertriglycéridémie, un faible taux de cholestérol HDL et de l'hypertension artérielle. Les maladies cardiovasculaires caractérisées par une dysfonction endothéliale sont le résultat clinique primaire du syndrome métabolique. De plus, les microparticules (MP), de petites vésicules membranaires libérées de la membrane plasmique des cellules activées et / ou apoptotiques ont été décrites comme étant impliquées dans la pathogenèse du syndrome métabolique car elles induisent une dysfonction endothéliale par la diminution du monoxyde d'azote (NO). D'autre part, des MPs générées à partir de cellules T apoptotiques sont capables induire une dysfonction endothéliale par la diminution de la production de NO. Cependant, les mécanismes par lesquels les MPs humaines induisent cette dysfonction endothéliale ne sont pas complètement élucidés. Ainsi, l'objectif de cette étude est d'étudier les mécanismes par lesquels les MPs humaines induisent une dysfonction endothéliale.

Mots clés

Syndrome métabolique, microparticules, maladies cardiovasculaires, dysfonction endothéliale, monoxyde d'azote, stress du réticulum endoplasmique, mitochondrie, stress oxydative.

Abstract

Metabolic syndrome (MetS) consists of a constellation of metabolic abnormalities such as central obesity, impaired fasting glucose, hypertriglyceridemia, low HDL cholesterol and hypertension. Cardiovascular diseases are the primary clinical outcome of MetS whereas endothelial dysfunction represents a primary disturbance in cardiovascular events. Recently, it has been shown that microparticles (MPs), small membrane vesicles released from the plasma membrane of activated and/or apoptotic cells, are involved in the pathogenesis of MetS by inducing endothelial dysfunction through the decrease of nitric oxide (NO) production. Also, MPs from apoptotic T cells induce endothelial dysfunction by decreasing NO production. However, the mechanism through which this endothelial dysfunction takes place is not completely elucidated. Thus, the objective of this study is to study the mechanisms through which human MPs induce endothelial dysfunction.

Key Words

Metabolic syndrome, microparticles, cardiovascular diseases, endothelial dysfunction, nitric oxide, endoplasmic reticulum stress, mitochondria, oxidative stress.