Universidade de Lisboa
Faculdade de Ciências da Universidade de Lisboa
Departamento de Biologia Animal

THE ROLE OF PERIPHERAL BLOOD MONONUCLEAR CELLS IN ATHEROGENESIS

Cláudia Simões Martins Bispo

Mestrado em Biologia Humana e Ambiente
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ABSTRACT

Atherosclerosis (ATH) is recognized as a chronic inflammatory condition and it is the leading cause of cardiovascular disease. Atherogenesis is characterized by the infiltration of low density lipoprotein (LDL) through the endothelial layer, and migration of activated peripheral blood lymphocytes (PBL) and monocytes (PBMN) that contribute to a pro-inflammatory state in specific locations. However, the functional interaction between immunity and LDL metabolism is still not fully understood. One hypothesis for the etiopathogeny of ATH may be associated with an ongoing inflammatory process caused by a pro-oxidant/anti-oxidant imbalance induced by metals such as iron (Fe) or copper (Cu). Interestingly, ceruloplasmin (Cp) is a multicopper oxidase with a relevant role in Fe metabolism and oxidation of LDL, but also an acute-phase protein involved in the inflammatory process.

Herein, we intended to study by flow cytometry the effect of putative pro-atherogenic immune stimuli on the expression of Cp at the surface of human peripheral blood mononuclear cells (PBMC), and search for a putative association with LDL oxidation. Additionally, a population of Familial Hypercholesterolemia (FH) patients was used as a clinical model of ATH to clarify the physiologic interplay between inflammation, Fe/Cu and lipid metabolism at systemic level in this disease.

The obtained results showed that higher cell surface expression of Cp was consistently observed in PBMN compared to PBL, in activated *vs* non-activated cells and in non-T cells *vs* T cells. Also, PBMC surface expression of Cp was differently modulated by several tested treatments. In particular, various modulators caused opposite effects on Cp expression of PBMN compared to PBL, suggesting a specific cell-type regulation for this protein. Specifically, it was observed that different sources of Fe might activate specific regulation mechanisms of Cp. Until now, due to technical limitations it was not possible to demonstrate an association between PBMC surface Cp expression and oxidation of LDL, but this issue should be addressed in future experiments. Of notice, IL-1β which was found to significantly increase PBMN surface Cp expression *in vitro*, was increased in serum from FH patients and positively correlated with total cholesterol and ApoB. However, the fact that no correlation was found between IL-1β and serum Cp (sCp)/oxidated LDL suggest that at systemic level, IL-1β may not be a contributing factor for oxidation of LDL by sCp. However, the role of PBMC surface Cp expression on LDL oxidation, namely in specific inflammatory states, remains to be established.

In summary, the results of this study demonstrate that specific pro-atherogenic conditions are involved in the modulation of Cp expression at surface of PBMC and thus, suggest a possible a role of Cp in ATH.

Key Words: Atherosclerosis - Ceruloplasmin - Mononuclear cells - Iron Metabolism

SUMÁRIO

A aterosclerose (AT) é a principal causa de doenças cardiovasculares (DCV). Muito embora esta doença tenha sido numa primeira fase considerada como uma simples deposição de lípidos, avanços substanciais nas áreas da ciência experimental permitiram revelar o importante papel da inflamação nesta patologia. Actualmente, a AT já não é reconhecida como uma simples consequência de envelhecimento considerando os factores de risco tradicionalmente aceites no seu envolvimento, mas antes como uma doença inflamatória crónica que pode ser convertida num evento clínico agudo por ruptura de placas ateroscleróticas e consequente trombose.

A formação da placa aterosclerótica (aterogénese) caracteriza-se por uma infiltração e activação precoce de monócitos e linfócitos (células mononucleares) que contribuem para um estado pró-inflamatório localizado em regiões de endotélio disfuncional. Adicionalmente, a passagem de lipoproteínas de baixa densidade (LDL) através da camada endotelial e a sua oxidação constituem eventos precoces da aterogénese. Contudo, os mecanismos fisiológicos envolvidos na interacção funcional entre as células imunitárias e a oxidação das LDL não estão completamente esclarecidos. Uma hipótese para a etiopatogenia da AT poderá estar associada à existência de um processo inflamatório contínuo relacionado com *stress* oxidativo induzido por metais, tais como ferro (Fe) ou Cobre (Cu). A ceruloplasmina (Cp) é uma proteína de fase aguda da família das multicobre oxidases com funções importantes no metabolismo do Fe, principalmente devido à sua actividade ferroxidásica. Para além disso, esta proteína apresenta potencial pró- e anti-oxidante, actividades relevantes no contexto do *stress* oxidativo característico deste processo.

O objectivo geral deste estudo foi investigar e caracterizar *in vitro* alguns dos mecanismos subjacentes à relação funcional entre inflamação, metabolismo do Fe/Cu e a homeostase lipídica, de forma a adquirir novos conhecimentos sobre a fisiopatologia da AT. Em particular, estudaram-os efeitos de *status* de Fe e Cu alterado, citocinas pró-inflamatórias e outros imunomoduladores na expressão de Cp à superfície de células mononucleares de sangue periférico (PBMC). Este estudo foi realizado através da utilização de citometria de fluxo, que possibilitou caracterizar a expressão da Cp membranar em subpopulações leucocitárias específicas de monócitos (PBMN) e linfócitos (PBL) de sangue periférico. Posteriormente, avaliou-se a possível associação entre a modulação da expressão de Cp membranar nas condições testadas e a oxidação de LDL. Adicionalmente, realizou-se a caracterização laboratorial de uma população de doentes diagnosticados com Hipercolesterolemia Familiar (HF), como um modelo clínico de AT. Neste contexto, pretendeu-se complementar os estudos *in vitro* procurando a existência de associações entre os parâmetros do metabolismo do Fe/Cu, lípidos e inflamação medidos a nível sistémico.

Os resultados obtidos através de citometria de fluxo mostraram consistentemente que a expressão de Cp à superfície de PBMN é maior comparativamente com PBL e que em ambas as populações as células activadas apresentam maiores níveis de expressão desta proteína que as suas

homólogas não activadas. Além disso, mostrou-se que dentro das subpopulações de PBL, são as células não-T que possuem maior expressão de Cp à sua superfície em comparação com as células T. Por outro lado, os resultados obtidos através das experiências de modulação mostraram que a Cp membranar das duas populações celulares em estudo é diferentemente regulada na presença dos diversos estímulos. Em condições de *status* de Fe alterado foi observado que a Cp à superfície de PBMC é diferentemente regulada nos dois tipos celulares testados, e parecem existir mecanismos de regulação específicos consoante o dador de Fe utilizado. Adicionalmente, a alteração do *status* de Cu não produziu modificações significativas na expressão de Cp à superfície de PBMC. Estas observações não corroboraram resultados obtidos anteriormente por outros autores e devem-se provavelmente ao curto espaço de tempo de incubação que não possibilitou visualizar o efeito de variação da concentração deste metal na estabilidade da Cp.

Em relação aos tratamentos com as citocinas e outros imunomodulatores observou-se de uma forma geral que a interleucina (IL)-1β, lipopolissacarídeo, e forbol 12-miristato 13-acetato induziram o aumento da expressão de Cp à superfície dos PBMN enquanto que a diminuíram nos PBL, sugerindo que a Cp membranar destas populações celulares poderá ser regulada especificamente devido às funções particulares e diferenças de cada tipo celular durante a resposta inflamatória. Os tratamentos com interferão-γ e IL-2 diminuíram a expressão de Cp à superfície de PBMN e PBL em incubações de longa duração, enquanto os tratamentos efectuados com os mediadores IL-6, IL-8, factor estimulador de colónia de macrófagos e o factor de necrose tumoral-α não resultaram variações na expressão membranar de Cp em ambos os tipos celulares. Provavelmente, a expressão de Cp à superfície de PBMC poderá ser nestes casos modulada em curtos espaços de tempo, pelo que as condições experimentais utilizadas não permitiram confirmar esta hipótese. A influência de espécies reactivas de azoto na expressão de Cp à superfície de PBMC foi igualmente testada realizando o tratamento com S-nitroso-N-acetilpenicilamina(dador de óxido nítrico), verificando-se que na presença deste tipo de condições de *stress* oxidativo são especificamente os PBMN que aumentam a expressão de Cp à sua superfície. Estes resultados sugerem que em estado pró-inflamatórios e de *stress* oxidativo a Cp expressa à superfície dos PBMN parece estar mais associado ao papel de proteína de fase aguda descrito para a forma circulante da Cp enquanto nos PBL esta proteína parece estar mais associada à regulação de *status* de Fe intracelular de modo a permitir a proliferação deste tipo de células após estimulação. Posteriormente, avaliou-se uma possível associação entre a indução de Cp à superfície de PBMC e oxidação das LDL no meio. Contudo, limitações técnicas impediram a confirmação desta hipótese neste trabalho. No entanto, a detecção de níveis de LDL oxidada (oxLDL) no sobrenadante de culturas em que Cp membranar de PBMN fora induzida por IL-1β, sugere a existência de potencial pró-oxidante na forma membranar de Cp. Esta observação carece contudo de confirmação após optimização de procedimento experimental utilizado.

Os resultados decorrentes da caracterização clínica do grupo de indivíduos diagnosticados com HF revelaram que estes doentes apresentam diferenças nos parâmetros relacionados com o metabolismo dos lípidos, consistentes com o seu diagnóstico molecular que mostra a existência de mutações genéticas em componentes do metabolismo lipídico. Nestes doentes foram ainda encontrados valores mais elevados de ferritina, oxLDL e IL-1β comparativamente com os indivíduos saudáveis. Além disso, nos doentes HF observou-se a existência de correlações positivas entre Cp circulante (sCp) e oxLDL, concordante com potencial pró-oxidante desta proteína, e entre sCp e proteína C reactiva - duas proteínas de fase aguda presentes em processos inflamatórios envolvidos na progressão desta doença. Foi também encontrada a correlação positiva de IL-1β com níveis de colesterol total e ApoB, parâmetros associados com risco aumentado de DCV. Contudo, o facto de não ter sido observada nenhuma correlação entre os níveis de IL-1β e sCp / oxLDL nestes indivíduos sugere que a nível sistémico a IL-1β parece não contribuir para o aumento de acção pró-oxidante da sCp. No entanto não é de excluir a possibilidade de um possível papel fisiológico de Cp expressa à superfície das PBMC a nível local nas placas ateroscleróticas.

Em conclusão, os resultados deste estudo demonstram que modulatores específicos de Fe/Cu e mediadores inflamatórios putativamente associados a estados pró-aterogénicos podem estar, pelo menos em parte, envolvidos na modulação da forma membranar da Cp em PBMC, sugerindo um possível papel da Cp à superfície dessas células durante a aterogénese.

Palavras Chave: Aterosclerose - Ceruloplasmina - Células Mononucleares - Metabolismo do Ferro

INDEX

Agradecimentos i
Abstract ii
Sumário iii
Index of Figures and Tables viii
Abbreviations x
Introduction 1
1. Atherosclerosis and Cardiovascular Disease 1
1.1 Inflammation and Atherosclerosis 2
1.2 Atherogenesis 4
1.3 Atherosclerotic Plaque Microenvironment 5
2. Oxidative Stress in Atherosclerosis 6
2.1 A link between the immune system, iron metabolism and oxidative stress: Ceruloplasmin 7
Objectives 9
Matherials and Methods 10
1. Population of study 10
2. Isolation of peripheral blood mononuclear cells 10
3. *In vitro* modulation of cell surface ceruloplasmin expression 10
3.1. Iron and copper status 11
3.2. Cytokines and other immunomodulators 11
3.3. Reactive nitrogen species 12
4. Immunophenotyping and Flow Cytometry analysis 12
5. Quantification of oxidized LDL in cell culture supernatants 13
6. Laboratorial characterization of Familial Hypercholesterolemic patients: a model to study atherosclerosis 13
7. Statistical Analysis 13
Results 15
1. Ceruloplasmin expression at surface of peripheral blood mononuclear cells 15
2. Regulation of surface ceruloplasmin expression by iron and copper status 15
2.1. Modulation of iron status 16
2.2. Modulation of copper status 17
3. Regulation of surface ceruloplasmin expression by cytokines and other immunomodulators 18
3.1. IFN-γ 18

3.2. IL-1β 20
3.3. IL-2 21
3.4. IL-6 22
3.5. IL-8 23
3.6. M-CSF 23
3.7. TNF-α 24
3.8. LPS 25
3.9. PMA 26
4. Regulation of surface ceruloplasmin expression by reactive nitrogen species 26
5. Study of putative association of GPI-Cp expression and LDL oxidation 28
6. Biochemical and immunologic characterization of FH patients as a model to study atherosclerosis 28
Discussion 30
Future Perspectives 40
References 41
Appendixes 49

INDEX OF FIGURES AND TABLES

Figures

Fig. 1 - Role of inflammation in all stages of atherosclerosis 1
Fig. 2 - Infiltration of LDL into the endothelium..... 4
Fig. 3 - Flow Cytometry analysis of surface Cp expression in non-treated PBMC 15
Fig. 4 - Effect on PBMC surface Cp expression after overnight incubation with Holo-Tf. 16
Fig. 5 - Effect on PBMC surface Cp expression after overnight incubation with Fe-NTA. 16
Fig. 6 - Effect on PBMC surface Cp expression after overnight incubation with DFO..... 17
Fig. 7 - Effect on PBMC surface Cp expression after overnight incubation with CuHis 17
Fig. 8 - Effect on PBMC surface Cp expression after overnight incubation with BCS 18
Fig. 9 - Effect on PBMC surface Cp expression after 1h incubation with IFN-γ 18
Fig. 10 - Effect of IFN-γ on activated and non-activated PBMC surface Cp expression after 1h incubation 19
Fig. 11 - Effect on PBMC surface Cp expression after overnight incubation with IFN-γ 19
Fig. 12 - Effect on PBMC cell activation after overnight incubation with IFN-γ..... 19
Fig. 13 - Effect on PBMN surface Cp expression after overnight incubation with IL-1β 20
Fig. 14 - Effect on PBL surface Cp expression after overnight incubation with IL-1β. 20
Fig. 15 - Effect on PBMN surface Cp expression and cellular activation after overnight incubation with IL-2 21
Fig. 16 - Effect on PBL surface Cp expression and cellular activation after overnight incubation with IL-2 22
Fig. 17 - Effect on PBMC surface Cp expression after overnight incubation with IL-6 22
Fig. 18 - Effect on PBMC surface Cp expression after overnight incubation with IL-8 23
Fig. 19 - Effect on PBMC surface Cp expression after overnight incubation with M-CSF..... 24
Fig. 20 - Effect on PBMC surface Cp expression after overnight incubation with TNF-α..... 24
Fig. 21 - Effect on PBMC surface Cp expression and cellular activation after overnight incubation with LPS..... 25
Fig. 22 - Effect on PBMC surface Cp expression after 2 h incubation with PMA.. 26
Fig. 23 - Effect on PBMN surface Cp expression after overnight incubation with SNAP 27
Fig. 24 - Effect on PBMN surface Cp expression after overnight incubation with Il-1β and SNAP 27
Fig. 25 - Effect on PBL surface Cp expression after overnight incubation with SNAP. 28

Tables

Table I - Risk factors for Development of ATH 2

Table II - Summarized list of inflammatory mediators involved in atherogenesis 6

Table III - Iron and copper treatments used to modify Fe/Cu status in PBMC culture 11

Table IV - Experimental conditions used to test the effect of cytokines and other immunomodulators in PBMC culture 12

Table V - Biochemical and immunologic characterization of controls and FH patients 29

Table VI - Significant correlations of serum Cp or IL-1β with biochemical parameters measured in FH patients 29

ABBREVIATIONS

Ab Antibody
ApoA1 Apolipoprotein A1
ApoB Apolipoprotein B
ATH Atherosclerosis
BCS Bathocuproine disulphonate
CHD Coronary heart disease
Cp Ceruloplasmin
CRP C-reactive protein
Cu Copper
CuHis Copper histidine
CVD Cardiovascular disease
DFO Desferrioxamine
EC Endothelial cells
ELISA Enzyme-linked-immuno-sorbent-assay
Fe Iron
Fe-NTA Iron-nitrilotriacetate
FH Familial Hypercholesterolemia
GPI Glycosylphosphatidylinositol
h Hours
HDL High density lipoprotein
HIF Hypoxia-inducible factor
ICAM-1 Intercellular Adhesion Molecule 1
IFN-γ Interferon gama
IL Interleukin
INSA Instituto Nacional de Saúde Doutor Ricardo Jorge
LDL Low density lipoprotein
Lp(a) Lipoprotein (a)
mAb Monoclonal antibody
MCP-1 Monocyte chemoattractant protein-1
M-CSF Macrophage colony-stimulating factor
MFI Mean fluorescence intensity
MI Myocardial infarction
min Minutes
mL mililiter
ng nanogram
NK Natural killer cells
NO Nitric oxide
oxLDL Oxidized low density lipoprotein
PBL Peripheral blood lymphocytes
PBMC Peripheral blood mononuclear cells
PBMN Peripheral blood monocytes
PMA Phorbol-12-myristate-13-acetate
RNS Reactive nitrogen species
ROS Reactive oxygen species
sCp secreted Ceruloplasmin
SMC Smooth muscle cells
SNAP S-nitroso-N-acetylpenicillamine
Tf Transferrin
Th T helper cells
TNF-α Tumor necrosis factor alpha
VCAM-1 Vascular cell adhesion molecule-1

INTRODUCTION

1. Atherosclerosis and Cardiovascular Disease

Cardiovascular disease (CVD) is the main cause of death in Western societies, being responsible for approximately 50% of all deaths [1]. Also, the economic and social difficulties caused by CVD are immense, constituting the greatest single cause of adult disability and a serious economic burden [2].

CVD is a collective term that comprises the clinical manifestations of coronary heart disease (CHD) and cerebrovascular disease, both of which have as a primary cause Atherosclerosis (ATH). [3]

The development of CVD involves genetic factors as well other acquired and modifiable risk factors. Smoking, hypercholesterolemia, diabetes mellitus and hypertension, among others are some of the main players in the development of this disease; however the major underlying cause leading to CVD is indeed ATH. [2, 4]

ATH is considered to be a complex multifactorial disease [5] that begins in the first decade of life, generally affecting the large arteries [2] as fatty streak lesions start to form in the artery wall. These early lesions are characterized by inflammation, lipid accumulation, cell death and fibrosis. Over time, they will mature and develop new characteristics as continued recruitment of mononuclear cells (Figure 1A), proliferation and migration of smooth muscle cells (SMC) occurs [2, 6]. This type of lesions, are then called atheromas or atherosclerotic plaques (Figure 1B) [7].

Clinical complications of ATH can arise from plaques causing flow-limiting stenoses, as significant narrowing of the arterial lumen occurs [8]. The most severe clinical events happen however after the rupture of a plaque (Figure 1C). This usually ensues when the combined efforts of the physical forces of blood flow and intensified inflammatory activation (that may lead to local proteolysis) disrupt the arterial wall [3, 6, 7]. These actions will eventually expose prothrombotic material from the interior of the plaque to the blood, triggering a thrombus that can cause sudden thrombotic occlusion of the artery at the site of disruption and the subsequent blockage of blood flow. In the heart, this leads to myocardial infarction (MI) and heart failure, whereas in the arteries that perfuse the brain, it can cause ischemic stroke and transient ischemic attacks [6].

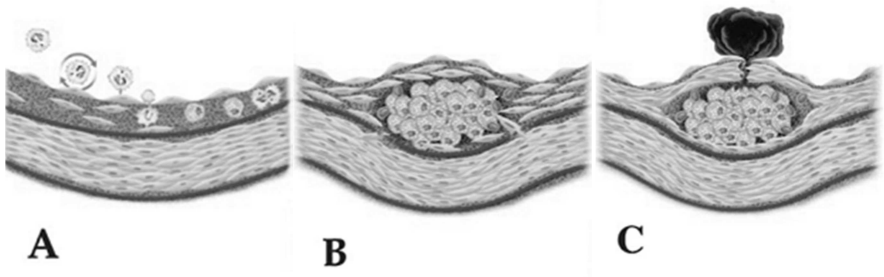

Fig. 1 - Role of inflammation in all stages of atherosclerosis. A) Leukocyte recruitment to the nascent atherosclerotic lesion. **B)** T lymphocytes join macrophages in the intima during lesion evolution. **C)** Alterations in extracellular matrix thin the fibrous cap, renders it weak and susceptible to rupture, triggering the thrombus that causes most acute complications of ATH. (From Libby *et al.* [9])

1.1 Inflammation and Atherosclerosis

During the last decades, experimental, clinical, and epidemiological studies have shed light on several key aspects related to this disease. Although ATH was formerly considered a bland lipid storage disease, the substantial advances in basic and experimental science have illuminated the pivotal role of inflammation in this disease [9], particularly the role of monocytes/macrophages and lymphocytes, and the defined series of changes that occur in the vessel during atherogenesis. It is now clear that ATH is not simply an inevitable degenerative consequence of ageing, but rather a chronic inflammatory condition that can be converted into an acute clinical event by plaque rupture and thrombosis [1].

Multiple independent pathways of evidence now identify inflammation as a key regulatory process that links multiple risk factors for ATH and its complications with altered arterial biology [10]. In this context, epidemiologic studies have identified many genetic and environmental risk factors (Table I) that seem to cause an impact on the inflammatory and immunological response of the organism [11]. Thus, cardiovascular risk factors are essentially injuries or insults to which the body adapts by continuously triggering a response throughout years of exposure [12].

Table I - Risk factors for Development of ATH
(From Greaves *et al.* [2])

Factors with a Significant Genetic Component
Elevated levels of LDL and VLDL
Low levels of HDL
Elevated lipoprotein (a)
Hypertension
Diabetes Mellitus
Male gender
Elevated levels of homocysteine
Elevated levels of hemostatic factors, e.g., fibrinogen
Metabolic syndrome
Insulin resistance
Obesity
Family history
Environmental Factors
Smoking
Lack of exercise
High fat diet
Infectious agents

This kind of compensatory response occurs preferentially at sites of hemodynamic strain [13], segments with low average shear but high oscillatory shear stress [14]. The normal homeostatic properties of the endothelium are then altered, and it becomes dysfunctional. Thus, the different forms of injury increase the adhesiveness of the endothelium with respect to leukocytes or platelets, as well as its permeability. They also induce the endothelium to have pro-coagulant instead of anticoagulant properties and to form vasoactive molecules, cytokines, and growth factors. If the offending agent or agents are not successfully dealt with by the immune system, and the inflammation as a chance to progress and continue indefinitely, the inflammatory response converts from a protective to an injurious response [15].

Such constant or repetitive injury can also stimulate the affected tissue to repair or wall off the damage by means of a fibroproliferative response, which, when excessive, diminishes the functional capacity of the tissue or organ and becomes part of the disease process. Because of this process, initially, the only cells thought to proliferate during expansion of atherosclerotic lesions were SMC [15]. However, as it is, most of the cells found in atheromas are blood-borne immune cells, and the remainder are then vascular endothelial and SMC [7]. Therefore, replication of monocyte-derived macrophages and T cells is probably equal importance during

the expansion of atheromas [16]. The ability of macrophages to produce cytokines (such as tumor necrosis factor α (TNF-α), interleukin (IL)-1 and transforming growth factor β), proteolytic enzymes (particularly metalloproteinases), and growth factors (such as platelet-derived growth factor and insulin-like growth factor I) may be critical in the role of these cells during the damage and repair that ensues as the lesions progress [15].

Many of the immune cells in the atheroma exhibit signs of activation and produce pro-inflammatory cytokines [7]. Activated macrophages express class II histocompatibility antigens that allow them to present antigens to T lymphocytes [17]. Thus, it is not surprising that cell-mediated immune responses may be involved in atherogenesis, since both CD4 and CD8 T cells are present in the lesions at all stages of the process [18]. This smoldering inflammatory state may lead to detectable blood levels of pro-inflammatory cytokines and other acute phase reactants. C-reactive protein (CRP) and IL-6 are elevated in patients with unstable angina and MI; high levels of these factors also predict a poor outcome [19, 20]. Furthermore, other inflammatory markers appear elevated in these patients, including IL-7, IL-8, soluble CD40 ligand, and the CRP-related protein pentraxin-3 [21-23].

The fact that several different inflammatory markers with different biological activities contribute to significantly increase the risk for CVD makes it unlikely that any of these particular markers actually causes the disease. Instead, the elevated levels may reflect an exacerbated inflammatory state in the atheroma, which promotes or causes plaque rupture, thrombosis, and ischemia [7]. In regard to possible causes of endothelial dysfunction, high levels of serum cholesterol are probably unique in being sufficient to drive the development of ATH in humans and experimental animals, even in the absence of other recognizable risk factors [11].

Free radical formation caused by cigarette smoking, hypertension, and diabetes mellitus causes focal endothelial activation in large- and medium-sized arteries [24]. Of these risk factors, smoking was shown to be one of the main factors in enhancing the risk of development of ATH, as it increases the lifetime risk in two-fold [25]. Likewise, the lack of exercise and the associated adiposity, in addition to a high intake of saturated fats and a low intake of certain vitamins, are commonly associated with an increased CHD risk in the general population [26]. Also, genetic alterations, as elevated plasma homocysteine concentrations that increases collagen production [27] and decreases the availability of nitric oxide [28], infectious microorganisms such as herpes viruses or *Chlamydia pneumoniae* and/or combinations of these, and other factors [29, 30], can contribute to atherogenesis [15].

The mechanism of action of these factors is, at least in part, thought to be associated with altered plasma levels of lipids and atherogenic lipoproteins. In fact, high levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol have consistently been shown to be connected with CHD risk. [26]

1.2 Atherogenesis

The early phase of atherosclerotic disease is best explained as an inflammatory response elicited by retention of lipoproteins in the arterial intima [7] at sites of dysfunctional endothelium, where disturbed flow dynamics cause modifications at the cellular and molecular level [14]. This occurs when plasma levels of the cholesterol-rich very low-density lipoprotein and LDL rise, and the lipoproteins infiltrate the artery wall to an extent that exceeds the capacity for elimination, and so they are retained in the extracellular matrix (Figure 2) [24].

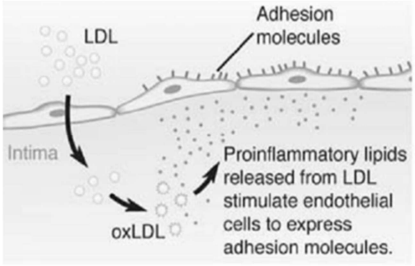

Fig. 2 - Infiltration of LDL into the endothelium. When it is retained in the intima, it can undergo oxidative modification (From Hanson *et al.* [7])

The oxidation of LDL is believed to occur locally within the arterial wall after retention [31]. Once LDL enters the vascular wall, the lipoproteins lose the protection from plasma anti-oxidants [32], thus their modification is promoted by oxidation, glycation (in diabetes), aggregation, association with proteoglycans, or incorporation into immune complexes [33, 34]. Presumably reactive oxygen species (ROS) produced by endothelial cells (EC), macrophages, proteins and several enzymes which occur in human atherosclerotic lesions have an important role at this stage [32].

EC and vascular SMC have been shown to oxidize LDL in culture [35-38], as have a number of enzymes [39-43] and proteins, such as ceruloplasmin (Cp) [44]. In some experimental situations, it was observed that administration of anti-oxidants can delay the progression of atherosclerotic lesions that develop in the face of hyperlipidemia, however human clinical trials have repeatedly failed to corroborate the concept that anti-oxidant vitamin therapy can improve clinical outcomes [9].

The oxidative stress generated within the intima [11] is a major cause of injury to the endothelium and underlying smooth muscle [45] and eventually evokes an inflammatory response in the arterial wall [11]. This response triggers the endothelial expression of adhesion molecules which cause blood cells rolling on the vascular surface to adhere at the site of activation [46] (such as vascular cell adhesion molecule-1 (VCAM-1) [47] and P-selectin [48]) and chemoattractant factors (such as monocyte chemoattractant protein 1 (MCP-1) and IL-8) that direct the migration and diapedesis of circulating monocytes and other leukocytes into the vascular intima of the arterial wall [49, 50] to neutralize or remove the offending agents [15]. Ultimately, this culminates in the formation of a fibrous plaque in that site [51], as illustrated in Fig. 1A and 1B. If the immune response is not successful, it can continue indefinitely. In doing so, the inflammatory response stimulates migration and proliferation of SMC that become intermixed with the area of inflammation to form an intermediate lesion [15].

The degree to which LDL is modified can vary greatly [45, 52] but once modified it can be internalized by macrophages by means of the scavenger receptors on the surfaces of these cells

[33, 34, 45, 53]. The internalization leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, resulting in their cellular activation and modification into foam cells [15]. Because these scavenger receptors are not down-regulated by increases of intracellular cholesterol levels, as it happens with the native LDL receptor, foam cells continually engulf modified LDL [54] until their eventual death, contributing to the amplification of the inflammatory process and the formation of the necrotic core found in advanced lesions [11].

Removal and sequestration of modified LDL are important parts of the initial, protective role of macrophages in the inflammatory response [52, 53] and minimize the effects of modified LDL on EC and SMC [15]. In fact, oxidized LDL (oxLDL) has numerous atherogenic properties on its own [55] and the bioactive phospholipids released by its modification can activate EC [56]. Furthermore, in addition to its ability to injure these cells [45], modified LDL is chemotactic on itself for other monocytes and can up-regulate the expression of genes for macrophage colony-stimulating factor (M-CSF) [57] and MCP-1 [58] derived from EC [15].

Thus, a vicious circle of inflammation, modification of lipoproteins, and further inflammation can be maintained in the artery by the presence of these lipids [15]. Specifically, mediators of inflammation such as TNF-α, IL-1 and M-CSF increase binding of LDL to endothelium and smooth muscle, and increase the transcription of the LDL-receptor gene [59].

1.3 Atherosclerotic Plaque Microenvironment

Within the inflamed arterial intima an altered cellular microenvironment is established, allowing the progression of ATH lesions [60, 61]. Interactions between macrophages foam cells, T helper (Th) 1 and Th2 cells eventually establishes the chronic inflammatory process [11].

A variety of different chemokines and cytokines mediates chemotactic recruitment of adherent monocytes and lymphocytes to the forming lesion [7], and can exert both pro- and antiatherogenic effects on each of the cellular elements of the vessel wall [11].

The ubiquitous monocyte, the precursor of macrophages in all tissues, is present in every phase of atherogenesis. Also, it's when T cells are activated by binding of antigens processed and presented by macrophages that the cell activation results in the secretion of cytokines, including interferon γ (IFN-γ) and TNF-α and β, that amplifies the inflammatory response. [6]

The unending entry, the survival, and replication of mononuclear cells in atherosclerotic plaques are dependent, in part, on factors such as M-CSF and granulocyte–macrophage colony-stimulating factor for monocytes and IL-2 for lymphocytes. Continued exposure to M-CSF allows macrophages to survive *in vitro* and possibly to multiply within the atherosclerotic plaques *in vivo*. In contrast, inflammatory cytokines such as IFN-γ activate macrophages and under certain circumstances induce them to undergo programmed cell death (apoptosis) [15].

But considering these effects in a global manner it was observed that the presence of M-CSF [62], TNF-α and IFN-γ [1, 32] in plaques changes expression of macrophages scavenger receptors, that will allow these cells to continually engulf modified lipoproteins leading to their accumulation in the cytoplasm until their death [6] and in this way become involved in the necrotic cores characteristic of advanced, complicated lesions [15].

When considering the role of inflammation in the development and progression of ATH it is also important to distinguish between local inflammation, shown in the activation of cells within the plaque microenvironment itself, and systemic inflammation, as evidenced by acute-phase proteins production and circulating pro-inflammatory mediators. Locally produced inflammatory mediators include the products of activated macrophages and T cells, such as TNF-α, IL-1β, IL-8, IL-18 and MCP-1, all of which are produced in response to modified forms of LDL and/or bacterial products. Systemic mediators and markers of inflammation include IL-6, IL-1β and CRP [2]. Table II lists some of the cytokines and inflammatory mediators that influence the development of atherosclerotic lesions in animal models of ATH [63].

Table II - Summarized list of inflammatory mediators involved in atherogenesis (From Greaves & Channon [2])

Pro-atherogenic mediators	Anti-atherogenic mediators
IFN-γ	IL-10
IL-18	IL-4
CD40–CD154	PPAR-γ (TZDs)
MCP-1 (CCL2)	TGF-β
IL-8 (CXCL8)	PDGF-β
IL-12	
IL-1β	
LTB4	
12/15-lipoxygenase products	

Abbreviations: IFN, interferon; IL, interleukin; LTB4, leukotriene B4; MCP, monocyte chemotactic protein; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; TGF, transforming growth factor.

2. Oxidative Stress in Atherosclerosis

In general, initiation and progression of ATH is best explained as an inflammatory response elicited by retention of lipoproteins in the arterial intima [7]. However, the mechanisms by which LDL is oxidized in the arterial wall are still not fully understood, but they might involve the cumulative effect of ROS or reactive nitrogen species (RNS) generated by inflammatory cells and enzymatic oxidation [64] during the response against the previous referred risk factors. For example, when in a hypertensive state the individual experiences a rise in the formation of hydrogen peroxide and free radicals such as superoxide anion and hydroxyl radicals in the plasma [45, 65]**.** These substances reduce the formation of nitric oxide (NO) by the endothelium [66], increase leukocyte adhesion and peripheral resistance [15].

Others authors proposed that the oxidative modification of LDL might be due to the existence of catalytically active transition metal ions [64] in the plaque microenvironment. The extensive LDL oxidation characteristic of atherosclerotic lesions may require a source of iron (Fe) or copper (Cu) as catalyst for the oxidation [67]. In fact, the interior of advanced human atherosclerotic plaques is a highly pro-oxidant environment containing redox-active Fe and Cu

ions, that *in vitro* induces lipid peroxidation [68, 69], but as Cu is more potent than Fe in catalyzing cellular LDL oxidation [70] it is possible it could account for the initiation of oxidation of LDL present in the arterial wall [71].

2.1 A link between the immune system, iron metabolism and oxidative stress: Ceruloplasmin

Cp is an abundant, blue plasma protein (metalloenzyme) which accounts for 95% of total circulating Cu in healthy adults [72]. Cp is a multifunctional protein with several functions that may play contradictory roles [73]. In fact, the protein is a multicopper oxidase and it possesses both anti-oxidant and pro-oxidant activities. It is also an acute-phase protein involved in the inflammatory process [67].

As a result of alternative splicing [74] there are two isoforms of Cp, one secreted mainly by the liver (sCp) and other anchored to the membrane by a glycosylphosphatidylinositol (GPI) group (GPI-Cp) in various cell types [75, 76], including monocytes [77]. Previously, it was demonstrated by our research group that GPI-Cp is also expressed in peripheral blood lymphocytes (PBL), reinforcing the intimate connection between Cp and the immune system [78] and its putative involvement during atherogenesis.

Cp is able to oxidise different substrates such as biogenic amines, phenols and Fe. On the other hand, Cp's anti-oxidant activity was also reported and it seems that this activity has a crucial importance in the inflammatory process and acute phase response [67]. This feature is evidenced not only by the capacity of Cp to act as a scavenger of superoxide anion radicals and other ROS [79], but also through the inhibition of Fenton reactions, by conversion of Fe^{2+} in to Fe^{3+} (a non-toxic form) due to the ferroxidase activity of Cp [67]. This contribution by Cp might help activated macrophages to drive Fe out of infected cells [80] and is thought to be an important mechanism in host defense by driving Fe homeostasis in a unfavourable way to the infectious organism [81]. Furthermore, Cp ferroxidase activity is thought to enhanced Fe efflux, by increasing the binding of Fe to transferrin (Tf), thus providing a negative Fe gradient from the perspective of the cell [82]. Observations in $Cp^{-/-}$ animals and in human patients with hereditary Cp deficiency, also supports this theory as it leads to an accumulation of Fe in the spleen, liver, and brain but with an overall systemic iron deficiency [83, 84]. Recently, it was reported that multicopper oxidases such as Cp are required for the Fe exporter ferroportin stabilization at cell surface, thus promoting cellular Fe efflux [85].

However, it is also known that Cp has the ability of oxidizing lipids through pro-oxidant activity. Ehrenwald *et al.* showed that Cp enhanced, rather than suppressed, the oxidation of LDL and that this activity was dependent on the integrity of its structure and its bound Cu [86]. Oxidative modification of Cp may result in conformational changes that dissociates the Cu

bound to Cp, thereby impairing its oxidase activity. In fact, free Cu may in turn promote oxidative reactions [87] with potential to cause various pathological effects, such as the decrease of NO bioavailability [86], which in turn could be an important factor in CHD since NO is responsible for inhibiting proliferation of vascular SMC, adhesion molecules expression and lipid oxidation[88].

Previous studies showed that levels serum Cp levels increase during pregnancy and in several pathological conditions including CVD [67]. Adelstain *et al.* were the first to correlate CVD and Cp by demonstrating that an increase in sCp was detectable after MI [89]. Afterwards, Kok *et al.* observed that individuals with the highest quantity of serum Cu were those who had four times higher risk of death from CHD [90]. It was also demonstrated that elevated serum LDL was associated with accelerated atherogenesis [91] and that individuals with reduced serum HDL had higher incidence of CVD. In this case, the individuals with the highest risk were those with low HDL and high Cp levels [92]. Consequently, it is believed that the contribution of Cp to the risk of CHD is not independent but rather depends on lipoprotein profile and perhaps other factors [93].

Regulation of Cp expression has been demonstrated in different cell populations [81, 94, 95]. Several cytokines and other factors induce sCp synthesis in hepatic cells, such as IL-1 and IL-6 [96], TNF-α and lipopolysaccharide (LPS). In cells of myeloid lineage, sCp synthesis was successfully induced *in vitro* by TNF-α in alveolar macrophages [97] and in monocytic cell lines by IFN-γ [81]. More recently, di Patti *et al.* reported the upregulation of Cp expression (both in the secreted and GPI-linked forms) by treatment with the pro-inflammatory cytokine IL-1β in a rat glial cellular model [94]. However, despite all these studies the physiologic functions of both Cp isoforms have not been elucidated with certainty.

Recently, it was shown that Cp and oxLDL colocalize in ATH lesions. Also, it was suggested that monocyte-derived macrophages might be the main Cp sources for LDL oxidation in the arterial wall, and that localize into the subendothelial space following endothelium activation [71].

In this study, we aim to improve our knowledge of the functional association between inflammation, lipid and Fe/Cu metabolism. Particularly, we expect to gain better insight into the role of Cp in the pathophysiology of ATH.

OBJECTIVES

The general goal of this thesis is to investigate *in vitro* and *in vivo* the underlying mechanisms relevant to the functional association of inflammation, lipid and Fe/Cu metabolism, in order to achieve a new understanding into the pathophysiology of ATH.

In order to achieve this main objective we specifically propose to:

1. Study in peripheral blood mononuclear cells (PBMC) from healthy individuals the regulation of cell surface Cp by altered Fe or Cu status, pro-inflammatory cytokines and other immunomodulators, and RNS;
2. Search for a putative association between PBMC surface Cp expression and oxidation of LDL;
3. Perform laboratorial characterization of Familial Hypercholesterolemia (FH) patients, as a clinical model of ATH;
4. Search for biochemical and immunological associations between the parameters measured in 3, in order to further understand the interaction of Fe/Cu, lipid metabolism and inflammation at systemic level.

In summary, with this study we expect to gain a better insight into the main mechanisms involved in the immune regulation of Fe/Cu metabolism related to the development of ATH, both at local and at systemic level.

MATERIALS AND METHODS

1. Population of study

Forty healthy individuals (27 females, 13 males; mean age 37.55 ± 12.36 years) and 40 FH patients (24 females, 16 males; mean age 42.85 ± 16.15 years) were included in the study.

The recruitment of healthy donors was rigorous and followed the subsequent criteria: absence of cardiovascular and lipid disorder; absence of medication and drug use; absence of drinking and smoking habits.

FH disease is one of the most common genetic disorders [98], and manifests itself by high levels of total and LDL cholesterol, with normal levels of HDL cholesterol and triglycerides. These individuals tend to develop premature ATH and have increased risk of CHD [99]. Serum samples from diagnosed FH patients were selected from the Portuguese Familial Hypercholesterolemia Study (EPHF) developed at Grupo de Investigação Cardiovascular, Unidade de I&D Departamento de Promoção da Saúde e Doenças Crónicas, Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA). These individuals also had associated a family history of hypercholesterolemia and CVD [54].

Blood and serum collection took place at INSA after all participants signed an informed consent form, approved by the ethical commission of the participating institution.

2. Isolation of peripheral blood mononuclear cells

Blood from healthy volunteers was collected in citrate CPT™ vacutainer tubes (BD, Germany) and PBMC were isolated according to the manufacturer's protocol. Briefly, tubes were centrifuged in the first 2 hours (h) after collection at 2800 rpm for 30 minutes (min) at room temperature to separate the PBMC from erythrocytes and granulocytes by the Ficoll inserted below a gel layer.

The resulting mixture of PBMC and plasma was recovered and then cells were pelleted (1500 rpm, 10 min). Residual erythrocytes were excluded using a lysis solution (10 min, 37 °C), and finally PBMC were washed with PBS. Assessment of cell viability was performed by Trypan blue exclusion method, using a Neubauer chamber before pelleted cells were cultured. Aliquots of plasma samples were stored at -80ºC for future laboratorial characterization.

3. *In vitro* modulation of cell surface ceruloplasmin expression

After isolation of PBMC from healthy volunteers, the cells were cultured in the presence/absence of a specific treatment depending on which condition to be tested, and

incubated in triplicates (n=3) at 37 ºC in a humidified incubator with 5% CO_2 atmosphere for each described time.

After the incubation period, PBMC were washed and pelleted (1500 rpm, 10 min) from the supernatants and ressuspended in Staining Buffer, the appropriate solution for immunophenotyping and Flow Cytometry analysis. The supernatants from the cultures were collected, alliquoted and stored at −80°C for future Western Blot analysis if needed.

3.1. Iron and copper status

Several compounds were used in order to increase or remove Fe or Cu ions from medium of PBMC culture. Increased Cu status was induced using Cu-Histidine (CuHis), which is a stable complex, while Cu depletion was achieved with Bathocuproine disulphonate (BCS), a specific Cu chelator. On the other hand, alterations of Fe status were induced on cell culture medium using Holotransferrin (Holo-Tf), a high affinity Fe saturated transferrin (responsible by the preferential and physiological way of Fe delivery in a wide range of cell types [100]). Also, a non-transferrin bound Fe compound was tested, 'free' Fe provided in the form of Fe-nitrilotriacetate (Fe-NTA, a stable complex). Fe depletion was achieved with Fe(III) chelator desferrioxamine (DFO).

The tested conditions were as follows:

Table III – Iron and copper treatments used to modify Fe/Cu status in PBMC culture

	Concentration tested	**Incubation time**
Holo-Tf	2,5; 5 mg/mL	20 h
Fe-NTA	10, 50, 100 µM	18 h
DFO	10, 50, 100 µM	18 h, 20 h
CuHis	10, 50, 100 µM	18 h
BCS	100, 200 µM	18 h

3.2. Cytokines and other immunomodulators

In order to study the effect of several putative pro-atherogenic stimuli on Cp expression at surface of PBMC, IFN-γ, IL-1β, IL-2, IL-6, IL-8, TNF-α (BD Pharmigen, USA) and M-CSF (R&D Systems) were used. Also, the inflammatory stimulators phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, USA) and LPS from *Escherichia coli* (Sigma-Aldrich, USA) were tested. Below are described the conditions used for each treatment:

Table IV – Experimental conditions used to test the effect of cytokines and other immunomodulators in PBMC culture

	Concentration tested	Incubation time
IFN-γ	1, 10 ng/mL	1 h, 18 h
IL-1β	20, 50 ng/mL	18 h
IL-2	10, 50 ng/mL	18 h
IL-6	10, 50, 100 ng/mL	18 h
IL-8	14.3, 71.5, 143 ng/mL	18 h
TNF-α	2, 12, 20 ng/mL	18 h
M-CSF	20, 50, 100 ng/mL	20 h
PMA	5 ng/mL	2 h
LPS	10, 100 ng/mL	20 h

3.3. Reactive nitrogen species

In the context of pro-atherogenic conditions present in the microenvironment of atherosclerotic plaques, ROS and RNS are very important to establish the pro-oxidant/anti-oxidant setting encountered at these sites.

To test the influence of specific RNS conditions on cell surface Cp expression of PBMC, cells from healthy donors were cultured overnight in the presence/absence of the NO donor S-nitroso-N-acetylpenicillamine (SNAP; Invitrogen, USA) at 100 and 500 μM concentrations.

4. Immunophenotyping and Flow Cytometry analysis

After the specific incubations, treated and non-treated PBMC were plated in 96-well round-bottomed microtiter plates (Nunclon, Denmark) at 5×10^5 cells/well and incubated for 15 min with AB-Human Serum, blocking the Fc-receptor binding sites for reduction of non-specific reactions. Afterwards, cells were stained for Cp using the rabbit anti-Human Cp (KOMA Biotech, Korea) as primary antibody (Ab) for 45 min, washed once with Staining Buffer and spun 250 *X g* at 4ºC for 5 min. Subsequently the cells were incubated for 45 min with the secondary Ab, a goat F(ab′)2 anti-rabbit FITC-conjugated (Rockland, USA) along with monoclonal Abs (mAb) CD45-PerCP, CD14-APC, CD3-PE and CD69-PerCP.

After staining, the cells were washed once with Staining Buffer, spun 250 *X g,* 4ºC for 5 min and ressuspended in the same solution, transferred to cytometer tubes and analyzed in a FACSCalibur Flow Cytometer (BD Biosciences, USA).

To determine Cp mean fluorescence intensity (MFI) in specific cell subsets, fluorochrome-conjugated mAbs against CD45, CD14, CD3 and CD69 were used. MAb CD45-PerCP allows for lymphocyte gating and combined with mAb CD3-PE allows the positive or negative selection of T cells. Also, mAb CD45-PerCP with CD14-APC were used for monocyte gating. In each case, mAb CD69-PerCP was used to gate activated and non-activated cells. All mAb were from BD Pharmigen, USA. Non-treated stained cells were used as negative control, to

determine autofluorescence in each assay. CellQuest (BD Biosciences, USA) was used for data acquisition, and measurement of Cp expression was performed using Weasel v.2.7.4 software.

5. Quantification of oxidized LDL in cell culture supernatants

PBMC were isolated from healthy donors and PBMN e PBL were incubated overnight with RPMI medium in the presence/absence of 20 ng/mL of IL-1β and human LDL (Sigma-Aldrich, USA) reconstituted in nitrogen-saturated solution, to certify the LDL was in its native state and minimize its spontaneous oxidative modification not related to the effect of Cp.

The concentration of oxLDL in the culture supernatants was measured by a sandwich Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) (oxLDL ELISA kit, K7810, ImmunDiagnostik AG, Germany) according to the kit protocol and measured spectrophotometrically with ELISA plate reader Milenia™ Kinetic Analyzer (Diagnostic Products Corporation, USA).

6. Laboratorial characterization of Familial Hypercholesterolemic patients: a model to study atherosclerosis

The groups of healthy individuals and FH patients was biochemically characterized through the determination of concentration levels of total cholesterol, HDL and LDL cholesterol, triglycerides, apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), Lipoprotein (a) (Lp(a)), Fe, Tf, ferritin, CRP and sCp, which were measured in serum samples (stored at -20ºC). Determination of lipid profile was performed by an enzymatic colorimetric method following the manufacturer's instructions in an automated chemistry analyzer (Cobas Integra 400, Roche). Determination of CRP, Fe, Ferritin, Tf, sCp levels were executed in the same equipment with respective method. All these analysis were performed at Unidade Laboratorial Integrada (INSA).

Additionally, the levels of oxLDL and IL-1β were quantified in serum samples using the following immunoenzymatic assays (ELISAs): oxLDL ELISA Kit (ref. K7810, ImmunDiagnostik AG, Germany) and BD OptEIA™ ELISA IL-1β Kit (BD, USA) respectively, following the manufacturers' instructions.

7. Statistical Analysis

All data analysis was performed using Microsoft™ Office Excel and SPSS v.17.

Results from each modulation experiment are presented as means ± Standard deviation in the respective figure. After the normality assessment was performed, to compared the means of two

related samples Student's t-test was used. In alternative it was employed the non-parametric test Wilcoxon.

When comparing the two independent samples from FH patients and control group, after normality assessment was performed the parametric Student's t test was employed. In alternative, the non-parametric test Mann-Whitney was used. Spearman correlation coefficient was used to analyze the relationship between the laboratorial characterization parameters of these two groups.

In all comparisons, the significance level to reject the null hypothesis was 5%, that is, *p*-values < 0.05 were considered statistical significant.

RESULTS

1. Ceruloplasmin expression at surface of peripheral blood mononuclear cells

Cp is usually described as a multifunctional protein with both anti-oxidant and pro-oxidant activities, being of crucial importance during the inflammatory process and acute phase response [67]. The intimate connection between Cp and the immune system [78] and possible role of Cp during atherogenesis is justified due to its existence in various cell types [76], including PBMN [74] and PBL [78]. Thus, in order to understand the possible physiological role of Cp expressed at the surface of specific leukocyte populations, this protein expression was analyzed at surface of PBMN and PBL isolated from healthy blood donors, using flow cytometry analysis.

Results obtained after overnight incubation of PBMC with culture medium alone (RPMI-1640 medium) showed consistently higher cell surface expression of Cp in PBMN compared to PBL (Figure 3A) and in activated *vs* non-activated cells (Figure 3B). Also, analysis of Cp expression at surface of PBL subpopulations, showed that non-T cells have higher surface Cp expression compared to T cells (Figure 3C).

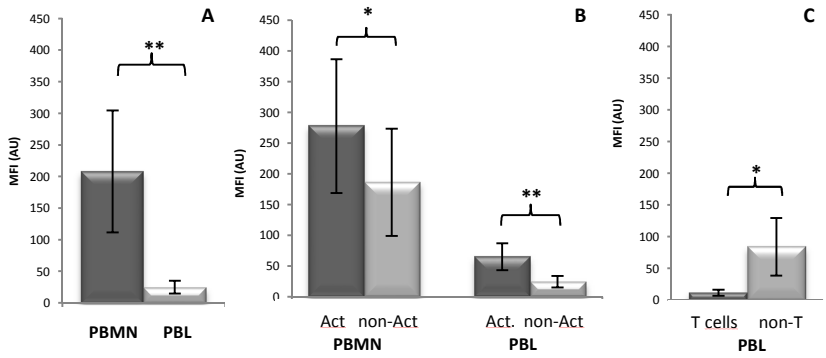

Fig. 3 –Flow Cytometry analysis of surface Cp expression in non-treated PBMC. A) Surface Cp expression in PBMN and PBL (n=16); **B)** Surface Cp expression in PBMN and PBL depending on their activation state (n=12); **C)** Surface Cp expression in PBL subpopulations (n=16), **(***p < 0.05, **p < 0.01).

2. Regulation of surface ceruloplasmin expression by iron and copper status

Human atherosclerotic plaques contain elevated levels of both Cu and Fe [68]. In order to test if Fe and/or Cu status influence the expression of surface Cp in PBMC, cells were treated in presence/absence of these metals under different experimental conditions.

2.1. Modulation of iron status

2.1.1. Holotransferrin

PBMC incubation with holo-Tf caused a 10% decrease in the percentage of Cp+ PBMN (data not shown), while a concomitantly non-statistically significant decrease in Cp surface expression was observed on this cells (Figure 4A).

On the other hand, PBL treated with holo-Tf showed upregulation of surface Cp expression in a dose dependent manner, being statistically significant for 5 mg/mL holo-Tf (Figure 4B) due to the upregulation of Cp expression in activated cells (Figure 4C). Also, a slight increase in the number of activated cells and Cp+ cells was observed in this cellular population (data not shown).

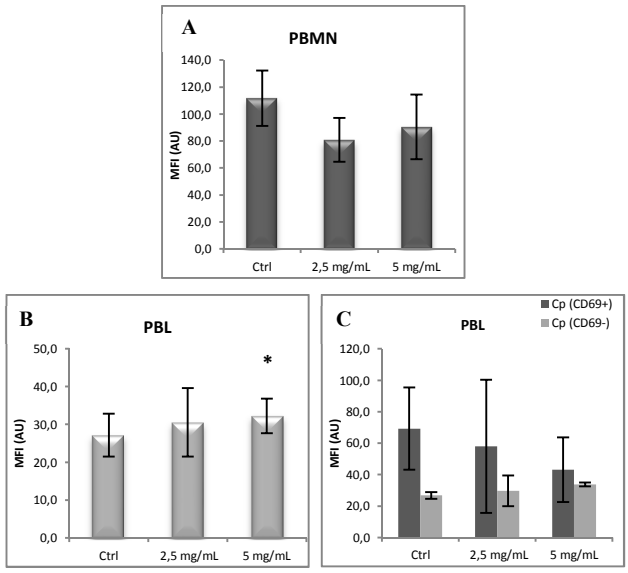

Fig. 4 - Effect on PBMC surface Cp expression after overnight incubation with Holo-Tf. Flow cytometry results of Cp expression at surface of **A)** PBMN, **B)** PBL and **C)** activated and non-activated PBL incubated overnight with Holo-Tf (Ctrl: non-treated cells, n=3, *p<0.05).

2.1.2. Iron-NTA

As observed in Figure 5, Fe-NTA treatment did not show any significant effect on Cp surface expression in PBMN and PBL (Figure 5A and 5B, respectively). In fact, only a trend to decrease in Cp expression at surface of PBMN could be observed, particularly at 100 µM Fe-NTA.

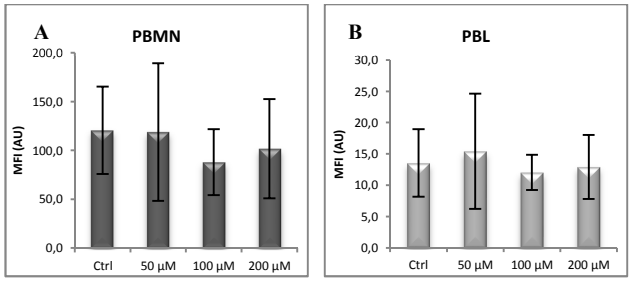

Fig. 5 - Effect on PBMC surface Cp expression after overnight incubation with Fe-NTA. Results of flow cytometry analysis of surface Cp expression in **A)** PBMN and **B)** PBL incubated overnight with Fe-NTA (Ctrl: non-treated cells, n=3).

2.1.3. Desferrioxamine

Incubation of PBMC with the Fe chelator DFO showed a trend to an increased surface Cp expression in both cell populations analyzed. In PBMN the upregulation of surface Cp expression was statistically significant at 100 μM DFO (Figure 6A).

In PBL, no statistically significant differences were observed between cells treated with DFO and non-treated. However, a trend to an increase of surface Cp expression in a dose dependent manner was observed (Figure 6B), with a concomitantly increase in the percentage of activated and Cp+ cells (data not shown).

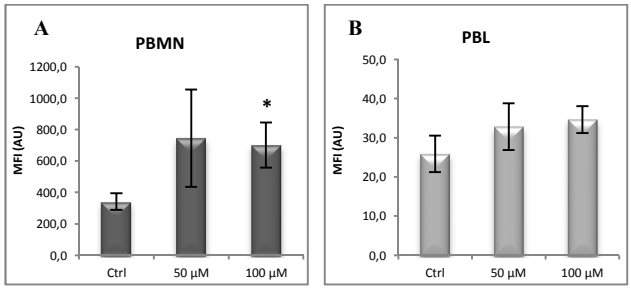

Fig. 6 - Effect on PBMC surface Cp expression after overnight incubation with DFO. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** PBL in cell cultures incubated overnight with DFO (Ctrl: non-treated cells, n=3, *p<0.05).

2.2. Modulation of copper status

Modulation of Cu status *in vitro* did not have any significant effect in surface Cp expression both in PBMN (Figure 7A) and PBL (Figure 7B). This finding was also observed in both PBMN (Figure 8A) and PBL (Figure 8B) when BCS Cu chelator treatment was tested.

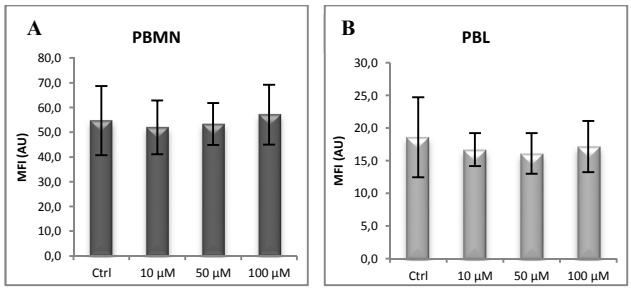

Fig. 7 - Effect on PBMC surface Cp expression after overnight incubation with CuHis. Results of flow cytometry analysis of surface Cp expression in **A)** PBMN and **B)** PBL incubated overnight with CuHis (Ctrl: non-treated cells, n=3).

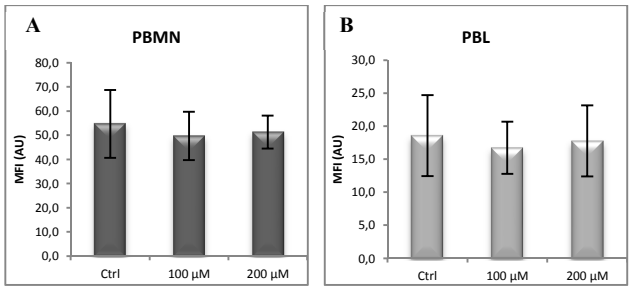

Fig. 8 - Effect on PBMC surface Cp expression after overnight incubation with BCS. Results of flow cytometry analysis of surface Cp expression in **A)** PBMN and **B)** PBL incubated overnight with BCS (Ctrl: non-treated cells, n=3).

3. Regulation of surface ceruloplasmin expression by cytokines and other immunomodulators

In order to test the hypothesis of Cp immunomodulation by pro-atherogenic stimuli, measurement of Cp surface expression of PBMC cultured in the presence of several cytokines and immunomodulators putatively associated to atherogenesis, was performed.

3.1. IFN-γ

IFN-γ is produced locally on atherosclerotic plaques [101] and promotes macrophage and EC activation. It also inhibits cell proliferation, collagen production, and cholesterol efflux [102]. Importantly, IFN-γ has been often considered a major pro-atherogenic cytokine [103].

In this study, PBMC incubated for 1 h in culture medium in the presence of IFN-γ showed a slight increase of surface Cp in PBMN (Figure 9A) and PBL (Figure 9B). However, these differences were not statistically significant.

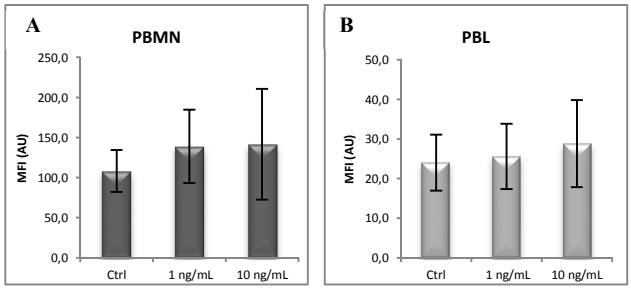

Fig. 9 - Effect on PBMC surface Cp expression after 1h incubation with IFN-γ. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** PBL incubated for 1 h with INF-γ (Ctrl: non-treated cells, n=3).

Observing Cp expression at surface of PBMN and PBL depending on their state of activation showed that while in PBMN the upregulation of surface Cp expression was due to both activated and non-activated cells (Figure 10A), in PBL the upregulation of Cp is mostly associated with activated cells (Figure 10B).

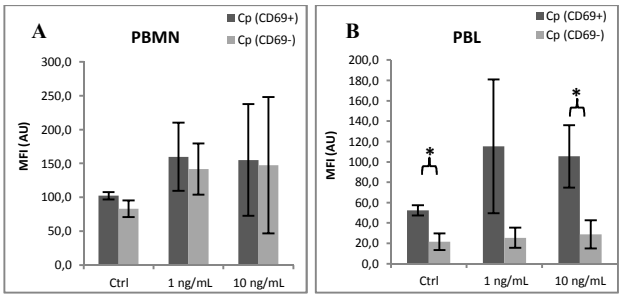

Fig. 10 - Effect of IFN-γ on activated and non-activated PBMC surface Cp expression after 1h incubation. Flow cytometry results of Cp expression at surface of activated and non-activated **A)** PBMN and **B)** PBL after 1 h incubation with INF-γ (Ctrl: non-treated cells, n=3).

On the other hand, in PBMC incubated in the same conditions but for a longer period of time (18 h), a decrease of Cp expression at cell surface was observed. Figure 11 shows the decreased expression in total PBMN (Figure 11A) and PBL (Figure 11B), being the decreased Cp expression in PBL statistically significant for 10 ng/mL (Figure 11B).

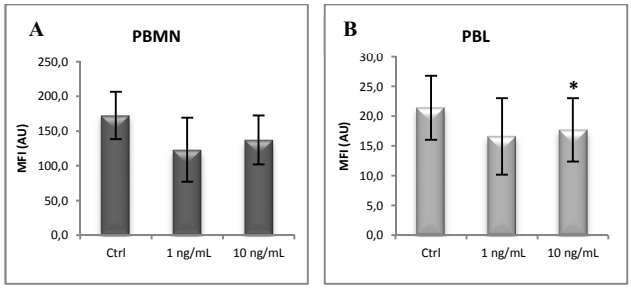

Fig. 11 - Effect on PBMC surface Cp expression after overnight incubation with IFN-γ. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** PBL incubated overnight with INF-γ (Ctrl: non-treated cells, n=3, *p<0.05).

Furthermore, in the presence of IFN-γ PBMN cell activation increased in a dose dependent manner (Figure 12A), while the percentage of activated PBL was not largely affected by this cytokine (Figure 12B).

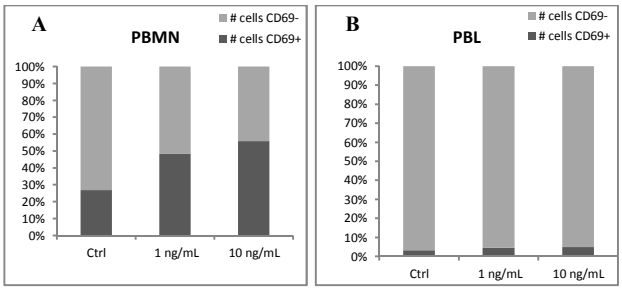

Fig. 12 - Effect on PBMC cell activation after overnight incubation with IFN-γ. Flow cytometry analysis of CD69+ expression at surface of **A)** PBMN and **B)** PBL in cell cultures incubated overnight with INF-γ (Ctrl: non-treated cells, n=3).

3.2. IL-1β

IL-1β is known as a lymphocyte activating and B-cell differentiation factor [104], but it was recently associated with CVD [105] and with ATH progression [106]. In this context, the effect of IL-1β on PBMC surface Cp expression was tested.

When PBMN were incubated with IL-1β at 20 and 50 ng/mL concentrations, an increase of surface Cp expression was observed in these cells (Figure 13A). Also, in this cell population, the number of activated cells increased with the treatment (data not shown), being surface Cp upregulation mostly associated with activated PBMN (Figure 13B).

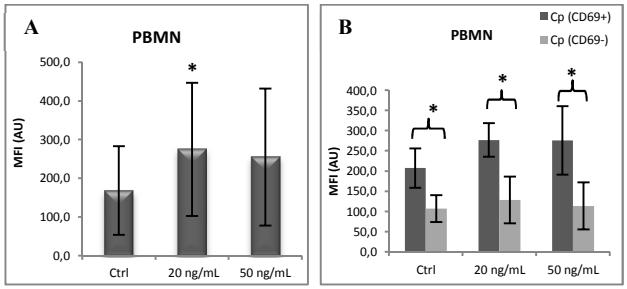

Fig. 13 - Effect on PBMN surface Cp expression after overnight incubation with IL-1β. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** activated and non-activated PBMN in cell cultures incubated overnight with IL-1β (Ctrl: non-treated cells, n=3, *p<0.05)

In total PBL, no significant effect of IL-1β on surface Cp expression was observed when comparing treated and non-treated cells (Figure 14A). However, surface Cp expression showed a tendency to decrease in a dose-dependent manner when analyzing activated *vs* non-activated PBL (Figure 14B). The percentage of Cp+ PBL was not affected by this interleukin and only a minimal increase in cell activation could be observed in PBL (data not shown). Regarding PBL subpopulations, a significant decrease of surface Cp expression was observed in non-T cells when in the presence of 50 ng/mL IL-1β (Figure 14C). Concomitantly, a small but steady increase in the percentage of activated non-T cells was observed along a small but constant decline in Cp+ cells was observed (data not shown).

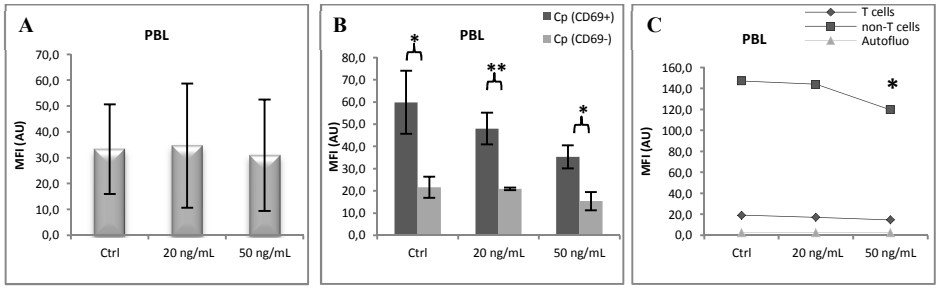

Fig. 14 - Effect on PBL surface Cp expression after overnight incubation with IL-1β. Flow cytometry results of Cp expression at surface of **A)** PBL, **B)** PBL depending on their activation state and **C)** PBL subpopulations incubated overnight with IL-1β (Ctrl: non-treated cells, n=3, *p<0.05, **p<0.01).

3.3. IL-2

IL-2 is a pro-inflammatory cytokine and a recognized growth factor for T and natural killer (NK) cells [107] involved in the proliferation and activation of these cells [108]. Recent studies showed serum IL-2 levels to be associated with atherosclerotic processes and a good indicator of the likelihood of an acute clinical event [109]. In this context, PBMC from healthy subjects were incubated overnight IL-2, to test for a possible modulation of surface Cp expression in these cells.

Our results showed that this treatment caused a progressive but non-statistically significant decrease in surface Cp expression in PBMN (Figure 15A). Similar effect could also be observed on PBMN regarding their activation state (Figure 15B), with both activated and non-activated PBMN showing a decrease of surface Cp expression. Furthermore, this modulation of Cp was observed along with a concomitantly decrease of Cp+ cell numbers (data not shown), while the percentage of activated PBMN increased slightly in a dose dependent manner (Figure 15C).

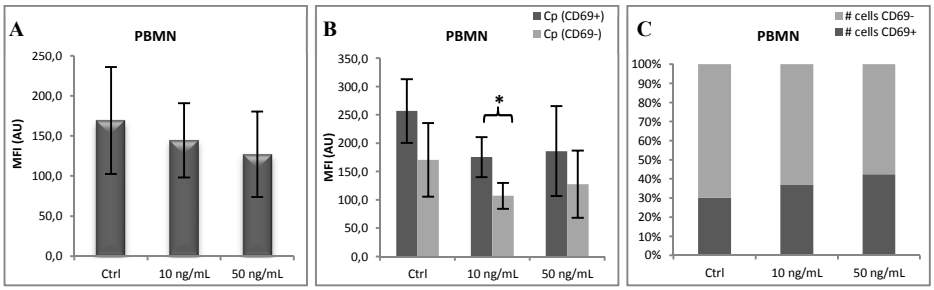

Fig. 15 - Effect on PBMN surface Cp expression and cellular activation after overnight incubation with IL-2. Flow cytometry results of Cp expression at surface of **A**) PBMN and **B**) activated and non-activated PBMN; **C**) Percentage of activated PBMN incubated overnight with IL-2 (Ctrl: non-treated cells, n=3, *$p<0.05$)

In PBL, the effects of IL-2 were mainly observed on non-T cells (data not shown), but one could notice that this cytokine significantly decreased surface Cp expression in a dose dependent manner in total PBL (Figure 16A and 16B). This Cp downregulation was concomitant with an increase in the percentage of activated PBL (Figure 16C) along with a decrease of the number of Cp+ cells (data not shown).

Analysis of PBL subpopulations also showed IL-2 significantly decreasing surface Cp expression in both T and non-T cells for the highest IL-2 concentration tested (Figure 16D). Finally, it was observed that IL-2 induced an increase in the percentage of activated non-T cells up to 40% (Figure 16E) whilst reducing by half the percentage of Cp+ cells in this specific subpopulation (Figure 16F).

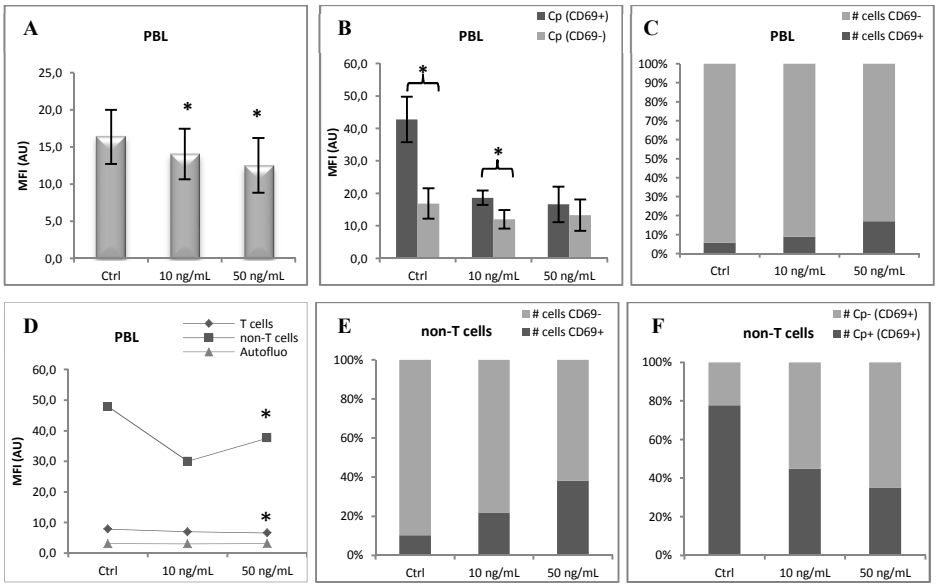

Fig. 16 - Effect on PBL surface Cp expression and cellular activation after overnight incubation with IL-2. Flow cytometry results of Cp expression at surface of **A)** PBL, **B)** activated and non-activated PBL, **C)** Percentage of activated PBL. **D)** Surface Cp expression in PBL subpopulations, **E)** percentage of activated non-T cells and **F)** percentage of Cp positive non-T cells incubated overnight with IL-2 (Ctrl: non-treated cells, n=3, *p<0.05)

3.4. IL-6

IL-6 is a multifunctional cytokine that plays a key role in the regulation of the synthesis of acute phase proteins [110] and is a major inducer of the systemic inflammatory response syndrome [111] with associated risk of CVD [112]. IL-6 was also observed to be increased in atherosclerotic arteries [113].

In this study, the results obtained showed that incubation with IL-6 did not cause any effects in the Cp expression at surface of PBMN and PBL, as can be seen in Figures 17A and 17B. Also, no change in the percentage of activated and Cp+ cells was observed after incubation with this modulator (data not shown).

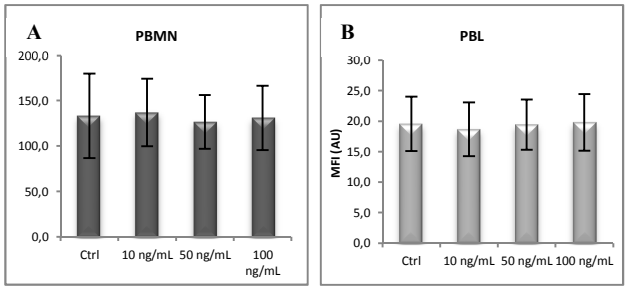

Fig. 17 - Effect on PBMC surface Cp expression after overnight incubation with IL-6. Results of flow cytometry analysis of surface Cp expression in **A)** PBMN and **B)** PBL incubated overnight with IL-6 (Ctrl: non-treated cells, n=3).

3.5. IL-8

IL-8 is a chemotactic cytokine that is released from stimulated EC [114], and it's able to activate and elicit leukocytes migration [115] (specifically neutrophils [116] and NK cells [117]) to sites of tissue injury to initiate local inflammatory response [118]. In this context, IL-8 could be seen as one of the initiation factors of atherogenesis after the first insults to the endothelium.

In order to test the hypothesis of IL-8 effect in surface Cp expression in PBMC, cells from healthy donors were incubated overnight with several concentrations of this interleukin. The results obtained showed no effect of IL-8 on surface Cp expression in PBMN in the tested conditions (Figure 18A and 18B). Similarly, no significant effect on surface Cp expression in total PBL was observed, despite a small decreasing trend (Figure 18C and 18D). Interestingly, this decrease on surface Cp expression in non-T cells was statistically significance for 71.5 and 143 ng/mL concentration of IL-8 (Figure 18E).

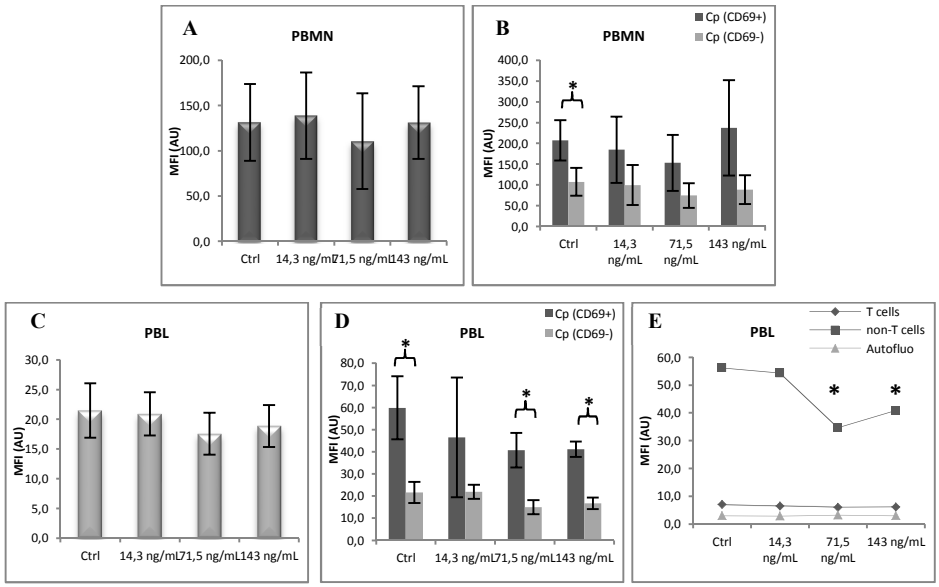

Fig. 18 - Effect on PBMC surface Cp expression after overnight incubation with IL-8. Flow cytometry results of Cp expression at surface of **A)** PBMN, **B)** activated and non-activated PBMN, **C)** PBL, **D)** activated and non-activated PBL and in **E)** PBL subpopulations incubated overnight with IL-8 (Ctrl: non-treated cells, n=3, *p<0.05).

3.6. M-CSF

M-CSF is described as a very important immunomodulator in innate immunity and inflammatory diseases [119]. It is synthesized by a variety of cell types, including EC. It is also able to promote survival, proliferation, and differentiation of mononuclear phagocyte lineages [119, 120]. In the atheroma formation, M-CSF is responsible for the differentiation of infiltrated monocytes into macrophages [121] and has been associated with upregulation of innate immunity mechanisms, including scavenger receptors and toll-like receptors [122].

In this context, we tested the effect of M-CSF on PBMC surface Cp expression. The results obtained showed no significant differences between treated and non-treated cells. One should however note that subtancial standard deviations were observed for both PBMN (Figures 19A) and PBL (Figures 19B).

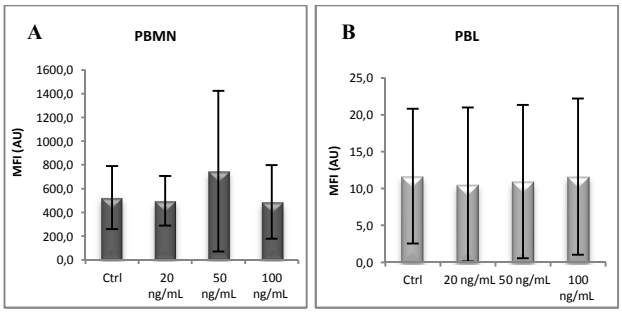

Fig. 19 - Effect on PBMC surface Cp expression after overnight incubation with M-CSF. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** PBL incubated overnight with M-CSF (Ctrl: non-treated cells, n=3, *p<0.05).

3.7. TNF-α

TNF-α is a pleiotropic pro-inflammatory cytokine that is produced by monocytes/macrophages and other cell types, including Th1 cells and NK cells. This cytokine is involved in a wide variety of immunological events, playing a key role in inflammation and is associated with the pathogenesis of ATH. In fact, TNF-α was recently shown to be involved in CVD susceptibility and progression, while its inhibition was associated with reduced ATH [123-125].

In this context, PBMC from healthy donors were treated with TNF-α overnight. The results obtained showed a trend to a progressive decrease of surface Cp expression in PBMN (Figure 20A) and PBL (Figure 20B). However, the differences between treated and non-treated cells were not statistically significant for both cases.

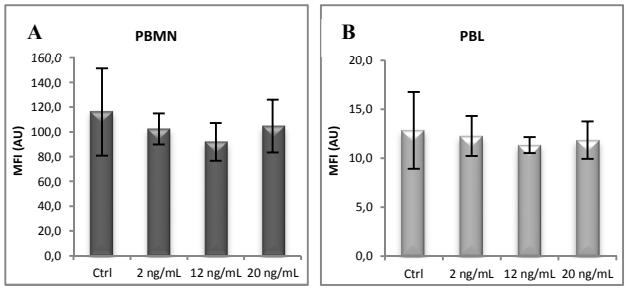

Fig. 20 - Effect on PBMC surface Cp expression after overnight incubation with TNF-α. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** PBL incubated overnight with TNF-α (Ctrl: non-treated cells, n=3).

3.8. LPS

As a classical endotoxin, LPS promotes an acute pro-inflammatory state, and in macrophages and B cells leads to secretion of various pro-inflammatory cytokines [126].

Incubation of PBMC with LPS for 20 h upregulated the expression of Cp at surface of PBMN (Figures 21A and 21B) while a trend to decreased surface Cp expression was observed for PBL (Figures 21C and 21D). These effects of LPS on Cp expression in PBMN and PBL were not statistically significant.

In addition, an increase of activated cells number was observed in both PBMN and PBL in the presence of this modulator (data not shown). Furthermore, non-T cells were the main subpopulation associated to this increase in activated cells (Figure 21E) as well as to the downregulation of surface Cp expression in total PBL (Figure 21F). On the other hand, non-activated non-T cells showed a slight and non-statistical significant upregulation of surface Cp expression in the same conditions (Figure 21F).

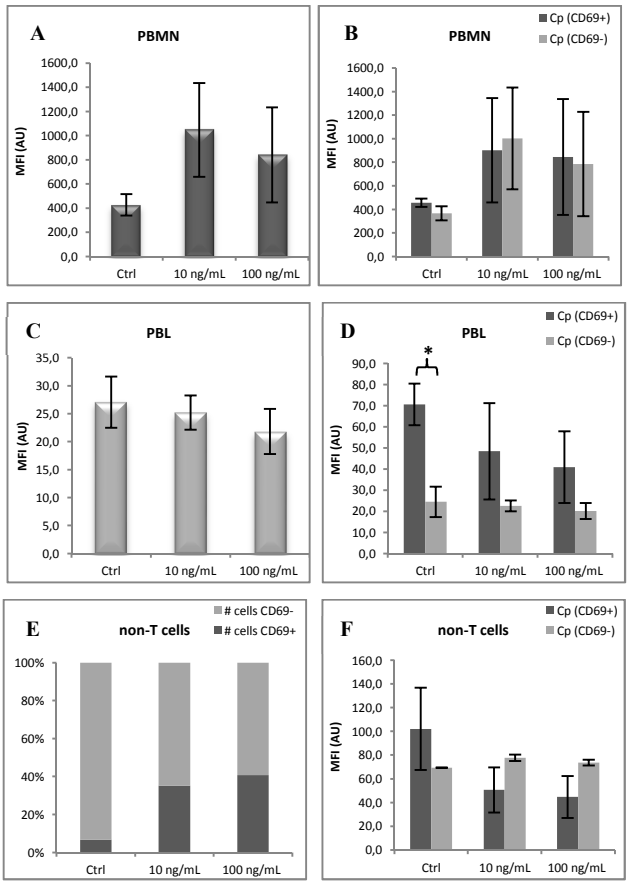

Fig. 21 - Effect on PBMC surface Cp expression and cellular activation after overnight incubation with LPS. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** activated and non-activated PBMN**, C)** PBL and **D)** activated and non-activated PBL; **E)** Percentage of activated PBL non-T cells and **F)** surface Cp expression of activated and non-activated non-T cells in PBMC culture incubated overnight with LPS (Ctrl: non-treated cells, n=3, *p<0.05).

3.9. PMA

PMA treatment is often employed in biomedical research as a way to induce immunologic activation in cell populations. As the previous results showed, activated PBMC express higher amounts of surface Cp compared to non-activated cells. In this context, the effect of this activatior on surface Cp expression in PBMC was tested.

Treatment of PBMC with 5 ng/mL of PMA for 2 h induced cell surface Cp expression in PBMN (Figure 22A). Also, activated PBMN were found to express significantly higher levels of surface Cp compared to non-activated cells in culture (Figure 22B). The effect of PMA on PBL caused a 40% increase of activated cells in culture (data not shown), but cell surface Cp expression showed a significant decrease compared to the non-treated cells (Figures 22C and 22D). In each of PBL subpopulations, the same trend could be observed but none of the effects showed to be statistically significant.

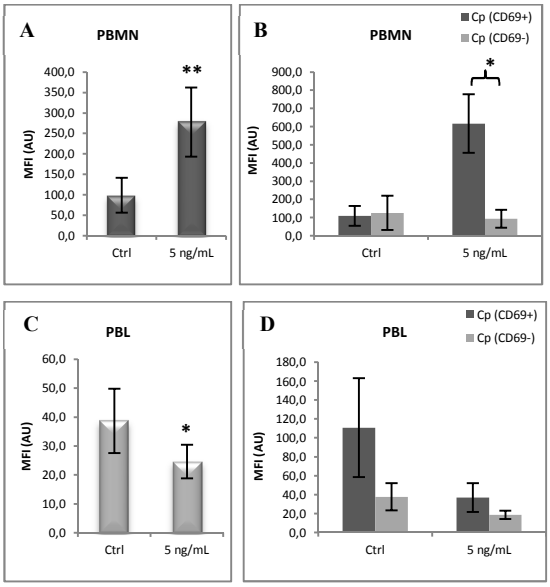

Fig. 22 - Effect on PBMC surface Cp expression after 2 h incubation with PMA. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** PBMN depending on their activation state, **C)** PBL and **D)** PBL depending on their activation state incubated for 2 h with PMA (Ctrl: non-treated cells, n=3, *p=0.05, **p<0.01).

4. Regulation of surface ceruloplasmin expression by reactive nitrogen species

NO is a relatively stable radical, however, when it reacts with superoxide (a common form of ROS generated during oxidative stress conditions, such as inflammation) generates peroxynitrite, a powerful oxidizing intermediate [71]. Immunohistochemical studies showed elevated levels of RNS in human atherosclerotic lesions [74, 80], suggesting that these may promote LDL oxidation *in vivo* [127, 128].

SNAP, a specific donor of NO, caused a significant increase of surface Cp expression in PBMN at concentration of 100 µM (Figure 23A). Also, a 2-fold increase in the percentage of activated PBMN was observed when in the presence of this compound (Figure 23B). This effect occurred concomitantly with a mild increase of the percentage of Cp+ PBMN (data not shown).

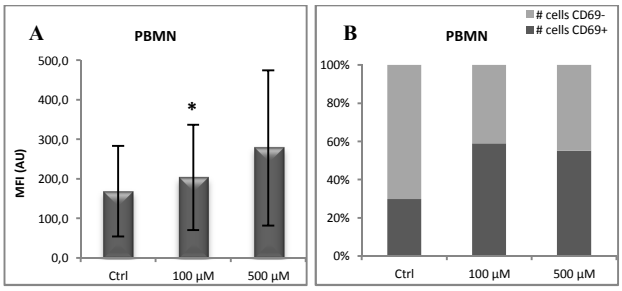

Fig. 23 - Effect on PBMN surface Cp expression after overnight incubation with SNAP. Flow cytometry results of Cp expression at surface of **A)** PBMN and **B)** PBMN depending on their activation state incubated overnight with SNAP (Ctrl: non-treated cells, n=3, *p<0.05).

When PBMN were incubated under the influence of both SNAP and IL-1β a slight synergistic effect on surface Cp expression could be observed. In fact, surface Cp expression in co-stimulated PBMN was higher than in non-treated PBMN or treated with only one of these modulators (Figure 24). However, the resulting co-stimulation surface Cp expression was shown to be statistically significant only when compared to surface Cp expression of PBMN treated with IL-1β alone.

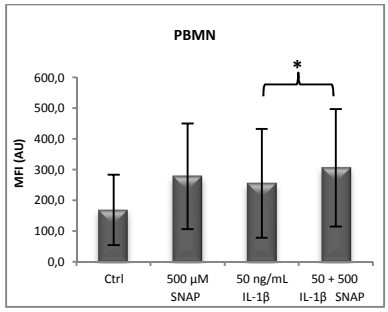

Fig. 24 - Effect on PBMN surface Cp expression after overnight incubation with Il-1β and SNAP. Results of flow cytometry analysis of surface Cp expression in PBMN incubated overnight with SNAP or IL-1β only, and IL-1β+SNAP (Ctrl: non-treated cells, n=3, *p<0.05).

In the case of PBL, SNAP did not induce any significant variations on Cp expression. A slight increase of Cp expression at surface of PBL was observed when treated with SNAP only (Figure 25A). However, in these conditions the percentage of activated and Cp+ cells did not change significantly (data not shown). Conversely, PBL co-stimulated by SNAP and IL-1β showed a lower yet (non-statistically significant) surface Cp expression than the one detected in non-treated cells and in cells incubated with only SNAP (Figure 25A and 25B).

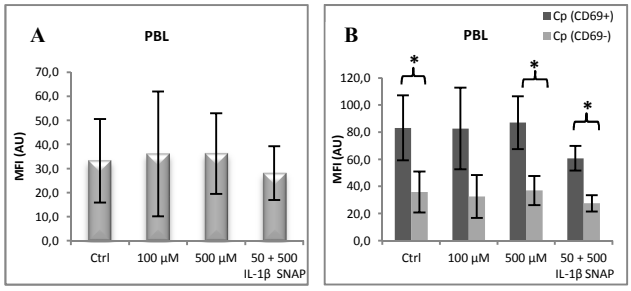

Fig. 25 - Effect on PBL surface Cp expression after overnight incubation with SNAP. Flow cytometry results of Cp expression at surface of **A)** PBL and **B)** aPBL depending on their activation state incubated overnight with SNAP and IL-1β+SNAP (Ctrl: non-treated cells, n=3, *p<0.05).

5. Study of putative association of GPI-Cp expression and LDL oxidation

The cell culture supernatants of PBMN and PBL overnight culture in the presence of IL-1β and native LDL were assayed in duplicate as described in Materials and Methods section. Concentrated aliquots of supernatants were still under the range of detection of oxLDL kit for most of the samples. The only samples in which oxLDL levels were detected were the ones where cells were incubated with IL-1β: in two conditions of PBMN incubated with native LDL and IL-1β, with an average concentration of 3.65 ng/mL of oxLDL.

6. Biochemical and immunologic characterization of FH patients as a model to study atherosclerosis

Serum samples of a total of 80 subjects were used for this study and constituted by two separate groups: the control group of 40 healthy individuals and the FH group comprised of 40 FH patients. All clinical, biochemical and immunologic data obtained from all these individuals is summarized in Table V. Descriptive and inference statistics were performed as described in section 7 of Materials and Methods section.

When comparing the Control group with FH group, significant differences could be observed in total cholesterol, LDL, triglycerides, ApoB and Lp(a). Immunological and Fe status parameters did not present statistically significant differences between these two groups, although ferritin, oxLDL and IL-1β showed increased levels in FH patients. The biochemical and immunologic parameters are summarized in Table V.

Table V - Biochemical and immunologic characterization of controls and FH patients. P values were indicated for statistically significant results, ns not statistically significant.

Parameters	Control		FH		p-value
	n	mean ± SD	n	mean ± SD	
Age (years)	40	37,55 ±12,36	40	42,85 ±16,15	
Female (%)	27	67,5	24	60,0	
Male (%)	13	32,5	16	40,0	
Total cholesterol (mg/dL)**	40	184,26 ± 30,04	40	258,43 ± 74,82	**<0,001**
HDL (mg/dL)**	40	56,18 ± 18,25	40	51,02 ± 15,78	ns
LDL (mg/dL)*	40	109,35 ± 21,19	40	186,15 ±69,70	**<0,001**
Triglycerides (mg/dL)**	40	101,03 ± 58,39	40	120,80 ± 50,98	**0,027**
ApoA1 (mg/dL)**	40	163,40 ± 40,28	40	156,43 ± 36,98	ns
ApoB (mg/dL)*	40	70,58 ± 12,55	40	111,60 ± 36,62	**<0,001**
Lp(a) (mg/dL)**	40	22,10 ± 19,39	40	57,65 ± 62,78	**0,03**
Iron (µg/dL)*	40	93,50 ± 34,08	40	91,50 ± 34,75	ns
Transferrin (mg/dL)*	40	284,15 ± 46,82	40	280,73 ± 56,53	ns
Ferritin (ng/dL)**	40	107,13 ± 106,61	40	131,36 ± 112,01	ns
CRP (mg/dL)**	40	0,36 ± 0,41	40	0,27 ± 0,30	ns
sCp (mg/dL)**	40	28,26 ± 9,06	40	26,67 ± 9,23	ns
oxLDL (ng/dL)**	40	118,83 ± 116,76	40	154,78 ± 131,06	ns
IL-1β (pg/dL)**	40	1,26 ± 1,19	40	4,00 ± 4,06	ns

*t Student test
**Mann-Whitney test
ns – non-significant

In order to search for possible correlations between the parameters measured, Spearman correlation coefficient was used. In the context of the current study, the significant correlations found in FH patients related to Fe/Cu, lipid metabolism and immunity/inflammation were: sCp negatively correlated with ferritin, and positively correlated with CRP and oxLDL. IL-1β was found to be positively correlated with total cholesterol and ApoB. These significant correlations are summarized in Table VI.

Table VI – Significant correlations of serum Cp or IL-1β with biochemical parameters measured in FH patients (n=40)

Parameter	*versus*	r	*p*
sCp	Ferritin	- 0,359	< 0,05
	CRP	0,375	< 0,05
	oxLDL	0,520	= 0,001
IL-1β	Total cholesterol	0,331	< 0,05
	ApoB	0,345	< 0,05

DISCUSSION

Several studies have been conducted in order to evaluate the impact of inflammatory factors, Fe/Cu status and oxidative stress in the development of ATH. The role of the circulating sCp isoform in this process has been addressed, but few studies pondered the putative importance of the membrane bound form of this protein (GPI-Cp) that appears to possess a more ubiquitous distribution [129] and is expressed in cells actively present in atherosclerotic plaque, such as PBL [78] and PBMN [77].

In order to study the underlying mechanisms relevant to the functional association of inflammation, lipid and Fe/Cu metabolism in ATH, one of the specific objectives of this study was to investigate the modulation of cell surface Cp in PBMC by putative pro-atherogenic stimuli. Herein, quantification of Cp expression at surface of PBMC was performed by flow cytometry, which allowed the measurement of this protein specifically in these two specific leukocyte subpopulations: PBMN and PBL. The obtained results showed consistently that PBMN express higher surface Cp compared to PBL. Also, analysis of T and non-T cells subpopulations showed that the highest surface Cp expression was observed in non-T cells, in concordance with previous observations [78, 130]. Furthermore, both activated PBMN and PBL, which are frequent in atherosclerotic plaques [6], showed a significantly higher surface Cp expression than non-activated cells. These results suggest that similarly to sCp, GPI-Cp may have a role in inflammatory response, since its expression was increased in activated cells and particularly in cell populations associated with innate immunity.

On the other hand, increasing evidence has pointed out the pathogenic roles of Fe during the development of ATH. In fact, Fe is present in atherosclerotic plaque at concentrations that substantially exceed those found in healthy arterial tissue, not being considered an inert component of plaque [68], part of the Fe accumulated is redox active and another portion remains as reservoir within lesions associated to specific proteins, such as ferritin and hemoglobin, but can potentially become reactive [131]. Cp has an essential role in Fe metabolism and, as a major ferroxidase in plasma, is required to catalyze the conversion of Fe^{2+} to Fe^{3+} for binding to Tf, the plasma Fe transporter [82]. Osaki *et al.* proposed that Fe released from cells in ferrous form was oxidized by Cp and bound to apo-Tf, creating a negative concentration gradient and thereby stimulating cellular Fe release [132].

In the current study, incubation with holo-Tf produced opposite effects on the two cell populations studied. Cp expression at surface of PBMN was decreased in the presence of holo-Tf compared to non-treated PBMN. In PBL, a significantly increase of Cp expression mostly due to non-activated PBL was observed. However, in activated PBL a trend to decrease of GPI-Cp expression was observed. These observations suggest that GPI-Cp expression is differently regulated by holo-Tf in both PBMN and PBL populations. Also, these differences might be due

to different physiological mechanisms of Fe management in these two types of cells. Monocytes/macrophages play an important role in Fe homeostasis, storing excess Fe in normal conditions and, during inflammatory response, these cells are responsible for restriction of Fe availability to local pathogens [133]. On the other hand, only recently was reported the expression of hepcidin (the most important protein in the regulation of Fe levels in the body [134]) in activated PBL [135]. Abrogation of hepcidin by siRNA unexpectedly significantly diminished lymphocyte proliferation. In this context, the decreased expression of surface Cp expression observed in PBMN, suggests that in the present experimental conditions PBMN might store the available Fe, through inhibition of Fe export and ferroxidase activity of Cp. Moreover, activated and non-activated PBL showed opposite trends of surface Cp expression in response to Fe treatment. Activated PBL showed decreased surface Cp expression, which supports the requirement of Fe for proliferation reported by Pinto *et al.* [135]. On the contrary, the increase of surface Cp expression of non-activated PBL in the presence of holo-Tf suggests these cells have a tendency to promote the ferroxidase activity of Cp and/or Fe release.

Incubation with Fe-NTA, a Fe compound more readily involved in Fe-driven redox reactions [136], did not produced any significant effects on Cp expression at surface of both PBMN and PBL. In fact, only a non-statistically significant decrease of this protein could be observed for PBMN. This appears to be in consensus with the results obtained during holo-Tf treatments, at least concerning PBMN surface Cp expression. The majority of PBMN in this culture were activated (data not shown), and under Fe overload conditions, results suggest that activated PBMN tend to promote Fe storage by inhibiting Fe release. Thus, in this context, diminished Cp expression could be one of the mechanisms involved. Previous reports support this hypothesis showing that incubation with Fe-NTA completely blocked Fe release in U937 cells [137]. Also, in C6 glioma cells, Fe overload induced by ferric chloride treatment showed no difference in expression of GPI-Cp mRNA, but induced a significant decrease in GPI-Cp protein [138]. On the contrary, the observation that this Fe loading treatment did not modulate surface Cp expression in PBL, suggests that these two different Fe sources, Tf-bound Fe (holo-Tf) or non-Tf-bound Fe, may underline a different regulation mechanism in these cells.

Decreased Fe status induced by Fe chelator DFO was previously reported to significantly increase GPI-Cp protein in C6 glioma cells by a translational regulation mechanism [138]. Sarkar *et al.* observed that in monocytic cell line U937 incubation with DFO markedly stimulated Fe release, in which all of the previous loaded Fe was removed within 15 minutes. These authors suggested that the development of a negative Fe gradient is an essential mechanism for Fe release from these cells [137]. In the present study, the observed results were consistent with these previous reports, since PBMC treated with Fe chelator DFO showed a higher percentage of Cp+ cells with a concomitant upregulation of surface Cp expression in a dose dependent manner in both PBMN and PBL. Thus, one can hypothesize that in conditions

of Fe deficiency, cells may promote intracellular Fe efflux by upregulation of surface Cp expression. These results may also be explained by the fact that DFO is an inducer of hypoxia-inducible factor (HIF)-1α, since Fe is required for constitutive HIF degradation [139]. Cp was the first positive acute phase protein reported to be regulated by HIF-1 activation [140]. In this context, the upregulation of surface Cp expression in PBMC treated with DFO could be explained by the non-degradation of HIF-1 and subsequent induction of Cp expression. This mechanism of Cp upregulation by HIF-1 could be very important in various pathologies, such as hypercholesterolemia and ATH, in which increased levels of HIF-1α in the atheroma plaque have been reported due to the hypoxic microenvironment in the plaque [13]. Also, oxLDL is itself an inducer of HIF-1α accumulation and activation in human macrophages [141]. This mechanism may be contributing for the consistent association of Cp levels with ATH and its localization in atherosclerotic plaques. Previous studies have indeed reported the existence of Fe [68] and Cp [71] accumulation in atherosclerotic plaques. However, in this study we observe that the increase of Fe status in PBMC cultures is followed in general by a tendency to decrease GPI-Cp expression in these cells. These observations indicate that Cp accumulation reported in atheromas may be resultant of HIF-1 activation due to hypoxic conditions or increasing levels of oxLDL in the plaque microenvironment other than to a putative Fe status regulatory mechanism of Cp expression.

Other metals are known to play important roles in vascular inflammation, such as Cu [67]. The most thorough data regarding how Cu status may affect Cp expression is related to sCp, partly due to its discovery decades before GPI-Cp [142]. Earlier studies, associated Cu deficiency with lower plasma Cp protein, as a consequence of Cu incorporation failure during its synthesis, resulting in the secretion of an unstable apo-Cp that is devoid of ferroxidase activity [143, 144] and with a half-life (5 h) considerably reduced when compared with holo-Cp form (5 days) [145]. Similarly, the influence of Cu limitations on GPI-Cp was recently studied and showed the rapid loss of GPI-Cp protein in spleen and, to a lesser extent in liver, of Cu deficient rats, which was observed with no reduction in GPI-Cp mRNA level [146] supporting that GPI-Cp apo-protein must present the same instability as the sCp apo-protein. Recently, it was also reported that treatment with Cu chelator tetrathiomolybdate consistently reduced sCp levels. The effect of this treatment also inhibited LPS-induced inflammatory responses in aorta and other tissues of mice, by inhibition of gene transcription of VCAM-1, ICAM-1, MCP-1, IL-6 and TNF-α, all of which related with vascular inflammation and ATH, suggesting that Cu lowering strategies could be of therapeutic value in ATH [147]. However, in the current study, alteration of Cu status did not cause any significant effects on surface Cp expression with either treatments of CuHis or BCS Cu chelator. These results may be explained by the fact that contrary to observations made by *in vivo* studies, the incubation period tested in our experiments

might not have been sufficient to allow the effects of Cp stability to be reflected on PBMC GPI-Cp expression.

Several inflammatory cytokines have been implicated in the pathogenesis of ATH. In this context, several pro-atherogenic cytokines and immunomodulators were incubated with human PBMC in order to study its effects on surface Cp expression.

Mazumder *et al.* reported for the first time that IFN-γ stimulated sCp production in monocytic cell line [81]. Later, it was also observed that induction of sCp in these conditions was subjected to translational silencing after 16 h incubation [148] by a structural element in the 3'-UTR of the Cp transcript [149] and that, similarly to sCp, a second monocytic Cp transcript was regulated in the same way [148]. Herein, distinct results of surface Cp expression after incubation with IFN-γ in the two tested conditions (1 h or overnight incubation) were observed. After 1 h incubation, a non-statistically significant increase of Cp expression was observed at the surface of both PBMN and PBL. In contrast, when PBMC were subjected to 18 h treatment with IFN-γ a decrease of surface Cp expression was observed (statistically significant in PBL). As previously reported, Cp protein synthesis ceases after 16 h by a mechanism of transcript-specific translational silencing. Nevertheless, the downregulation of surface Cp expression observed in this study could not to be explained by this mechanism. However, this significant decrease could be a result of Cp levels returning to its basal expression in order to inhibit deleterious effects of excessive Cp, after its maximum levels were reached possibly between 1 h and 16 h after stimulation by IFN-γ.

The multifunctional and pro-inflammatory cytokine IL-1 affects nearly every cell type, often in concert with other cytokines or other mediators [150]. IL-1β is of central importance in the development of diseases but also in normal homeostasis such as metabolism, hematopoiesis, renal and hepatic function [151, 152]. It was also one of the first cytokines to be considered instrumental in the propagation of vessel wall inflammation during atherogenesis, due to its role in the formation of early lesions [153, 154], maintenance of an inflammatory state [155, 156] and involvement in advanced plaque formation. Furthermore, its involvement in the destabilization and plaque rupture was reported [157]. These features are further supported by the observation that IL-1β serum levels are associated with a history of dyslipidemia and unstable angina [105] as well the the protective effect of IL-1β deficiency in ATH severity observed in apoE deficient mice [106]. Also, several IL-1β gene polymorphisms have been found to be associated with MI [158]. IL-1β was previously reported to induce Cp mRNA in hepatocytes [159] and secretion of sCp in astrocytes [160]. Later, di Patti *et al.* showed that IL-1β was able to induce GPI-Cp and ferroportin mRNA expression in glial cells, increasing Fe efflux in these cells in culture [94].

In the present study, PBMN treated with IL-1β showed a statistically significant upregulation of surface Cp expression, while in activated PBL and non-T cells a progressive decrease dependent on IL-1β concentration was observed. These results show that IL-1β has opposite effects on surface Cp expression in both PBMN and PBL, which may be related with specific physiological roles and regulation mechanisms of these cell types during the inflammatory response. The priority of activated PBL response in inflammatory conditions is to proliferate and, as stated before, these cells require Fe to do so. Thus, one can suggest that in these conditions Fe export could be inhibited in activated PBL and GPI-Cp diminished expression could be a consequence or a means to that inhibition, with shedding of GPI-Cp being a possible mechanism. On the other hand, the significant Cp upregulation observed in PBMN might be a result of Cp being an acute phase protein and its bactericidal activity induced by inflammatory status.

Previously, it was shown the incubation with pro-inflammatory cytokine IL-2 (produced largely by stimulated CD4 T cells and pro-inflammatory Th1 cells) further stimulates the cell growth and differentiation of PBL [53, 109]. Recent data from Elkind *et al.* also showed that serum levels of IL-2 were strongly associated with carotid artery intima-media thickness (a predictor of stroke and vascular disease) in a population with asymptomatic or subclinical state of ATH. These authors suggested that IL-2 may provide a better index of earlier atherosclerotic processes, while other commonly used markers - such as CRP - provide a better measure of later processes and the likelihood of an acute clinical event [109]. Herein, the treatment of PBMC with IL-2 resulted in a non-statistical significant decrease of surface Cp expression in PBMN, concomitant with a slight increase of activated cells. In PBL, this cytokine significantly decreased total PBL surface Cp expression in a dose dependent manner, while increasing the percentage of activated PBL. Also, it was observed that IL-2 activated a larger amount of non-T cells than T cells, whilst significantly decreasing their Cp expression. These results suggest that PBMC GPI-Cp may not be a contributing factor during the initial phase of atherogenesis in which IL-2 would be involved, particularly in activated PBL where the decreased GPI-Cp expression corroborates the hypothesis of inhibition of Fe efflux to promote their proliferation after inflammatory stimulation [135]. It would be important to evaluate the effects of IL-2 in shorter incubation periods, because similarly to what occurred with IFN-γ treatment, the present experimental conditions may not be ideal to detect faster cellular responses in Cp expression.

On the other hand, IL-6, a classical acute phase cytokine and a recognized pro-atherogenic factor secreted by T cells and macrophages to stimulate immune response [161], could represent an important mediator of surface Cp expression in PBMC during atherogenesis. However, in the present study it was observed IL-6 did not modulate surface Cp expression in both cell populations tested. This observation does not corroborate previous studies where was reported IL-6 increased levels of sCp protein in hepatoma cell line Hep3B [162] and had little effect on

sCp mRNA in HepG2 cell line [159]. However, it is in consonance with other report in monocytic cell line U937 treated for 24 h with IL-6 [81]. These results suggest that while no effect on surface Cp expression was observed after an overnight incubation with IL-6, it does not refute the possibility of IL-6 modulating Cp expression earlier during this treatment. Indeed, a possible increase in Cp expression at PBMC surface may have not been detected at the experimental conditions used during this study. In fact, Daffada *et al.* had previously reported that the peak of Cp mRNA in stimulated HepG2 occurred 4 to 8 h post-stimulation [159]. Thus, shorter incubation periods should be attempted in future studies in order to address the putative immediate effects of IL-6 in GPI-CP expression.

The influence of IL-8 on Cp expression at surface of PBMC was also tested. Since it is also considered a pro-atherogenic factor [161] locally produced in atherosclerotic plaques, and being a chemotactic cytokine [2], it was hypothesized this modulator could constitute a key initiation factor and/or be able to amplify the inflammatory response during atherogenesis. However, IL-8 had no effect on Cp expression at surface of PBMN. In total PBL, a non-statistically significant decrease in Cp expression dependent on IL-8 concentration was observed, while in non-T cells the decrease was statistically significant for the highest IL-8 concentration tested. These results suggest that similarly with other pro-inflammatory cytokines tested, the observable decrease in Cp expression might be a consequence of PBL proliferation after activation. In this case only non-T cells, which include NK cells, were observed to be significantly stimulated by IL-8, as expected [117]. The fact that IL-8 effect was only statistically significant at high concentrations is suggestive of non-T cells involvement in atherogenesis when such conditions are encountered in plaque microenvironment.

On the other hand, surface Cp expression in PBMC after M-CSF treatment was not significantly modulated. Technical alterations to the protocol may have hampered the collection of results, as the recovered cells were in much less quantity than in all other experiments (only 10%). Also, a non-specific interaction with platelets in culture could be occurring because during data acquisition, a percentage of unspecific cellular components could be observed in the plot. Indeed, the PBMC separation protocol does not eliminate platelets completely from the sample, and this particular chemokine promotes platelet adherence and platelet activation [163].

TNF-α, one of the markers of inflammation that is variably expressed *in situ* in vulnerable and unstable plaques [123] has been previously described as an inducer of hepatic Cp synthesis [97]. Considering the role of this immunomodulator on Fe metabolism (decreases Fe release, while increasing Fe uptake and storage within ferritin in monocyte/macrophages [164, 165]) and its direct influence in Fe-mediated ROS production [166], it was anticipated that TNF-α could be a modulator of Cp expression. However, TNF-α treatment did show any significant effect on surface Cp expression in both PBMN and PBL. This result may indicate that similarly with some of our previous results, the inexistence of Cp modulation in the present experimental

conditions may be explained by a limitation to measure Cp expression at surface of PBMC at the proper time, observing only the basal Cp expression of these cells. Thus, in order to test this hypothesis shorter incubation periods should be performed in future experimental designs to be tested.

Moreover, LPS - a known exogenous pyrogen that promotes an acute pro-inflammatory state with the consequential secretion of pro-inflammatory cytokines by many cell types (especially in macrophages and B cells) [126] - was tested as putative modulator of surface Cp expression in PBMC. As Cp is also an acute phase protein [95], it was expected the increase of sCp expression reported by Fleming *et al.* when different rat tissues were submitted to a dose of LPS endotoxin. The authors observed an increased content of sCp mRNA in the liver and also in the lung (specifically in alveolar macrophages) and, as sCp mRNA was not consistently detected in other extrahepatic tissues, it was concluded that the endotoxin effect was tissue specific [97]. In the present study, it was observed that incubation with LPS also modulated surface Cp expression in PBMC. In the case of PBMN, in spite of considerable Cp upregulation observed, the variation was not statistical significant, which could be an artifact of small sample size. However, this observation suggest that similarly to sCp, PBMN surface Cp might play a role in host defenses during acute phase responses, through bactericidal and ferroxidase activity. Studies showing that hepcidin synthesis in macrophages is upregulated in response to LPS, and inflammation [134] resulting in the downregulation of ferroportin and subsequently macrophage retention of intracellular Fe [167], support that the increase of surface Cp observed in this condition might be more related with its bactericidal activity than to enable Fe efflux. Inversely, LPS treatment in PBL increased activated cells in the population with a concomitant trend to non-statistically significant decrease of surface Cp expression dependent on concentration of LPS. Again, this observation on activated PBL may be a consequence of their Fe-dependent proliferation, in which PBL inhibit Fe efflux in order to promote their proliferative activity after stimulation. Such observation was also seen in previous treatments with other pro-inflammatory cytokines that induced PBL activation, reinforcing the hypothesis of inhibited Fe efflux and subsequent diminished Cp expression levels in order to favor activated PBL proliferation. A possible mechanism for diminishing GPI-Cp levels at cell surface could be by protein shedding, in order to quickly respond to such stimuli. However, measurement of this protein in the extracellular medium could not be performed due to time constraints, but it should be assessed in the near future to explore this hypothesis. Another interesting observation made during this treatment was that non-activated non-T cells actually show increased surface Cp expression compared to their activated counterparts. Despite the fact that this result lacks statistical significance, further investigations should be performed in order to understand the significance of this observation, and if implies different regulation of Fe content in these cells or another role altogether.

On the other hand, treatment of PBMC with PMA for 2 h showed very similar effects to the ones observed for LPS treatment. A statistically significant induction of surface Cp expression in response to PMA was observed for PBMN, while in PBL a significant decrease in their surface Cp expression was found. The same hypothesis regarding the functional role of surface Cp in both populations after incubation with LPS can be suggested here. In fact, similarly the current knowledge allows to hypothesize that increased Cp levels in PBMN might be related to its bactericidal activity as an acute phase protein, while in PBL the decrease in Cp expression could be caused by the need of these activated cells to proliferate and thus their need to inhibit Fe efflux from intracellular stores. In order to investigate such hypothesis, in future experiments Fe efflux/influx should be also measured in these cell cultures, and further analysis of culture supernatants should be crucial to demonstrate if protein shedding is involved in the rapid and significant downregulation of PBL GPI-Cp.

Given the previous reports of RNS involvement in ATH, the putative effect of this compounds in surface Cp expression was also tested. In fact, NO released by functional EC of the artery wall [168] is critical in vascular homeostasis [169] playing in overall, a protective role [170]. In spite of NO being a relatively stable radical, it reacts with locally produced ROS during the increasing oxidative stress generated in plaque microenvironment, especially with superoxide, to form peroxynitrite [171]. Peroxynitrite is a highly cytotoxic form of RNS [172], and has been observed in many inflammatory diseases, where it showed the ability of directly or *via* its reaction products, oxidize LDL and modify other proteins, as well releasing Cu ions by destroying Cp [173]. In the present study, RNS putative influence in surface Cp expression was tested by incubating PBMC with SNAP, a specific NO donor after which NO is readily oxidized. This treatment caused a significant increase of surface Cp expression in PBMN and doubled the percentage of activated cells, while in PBL no significant changes were observed. This observation suggests that oxidative stress generated by SNAP supports the previous hypothesis that Cp at surface of PBMN might have a more active role in extreme conditions as an acute phase protein, or in this case as anti-oxidant compound, compared to Cp expressed by PBL. The effect of SNAP on releasing Cu ions from holo-Cp was not directly addressed and the expected Cp downregulation by diminished stability of apo-Cp was not observed but, as previously discussed in BCS treatment, the incubation period tested here may have not been sufficient to detect the instability effect of apo-Cp on Cp expression levels. Also, when PBMN were co-incubated with SNAP and IL-1β, a synergistic effect could be observed, as inflammatory state and oxidative stress was induced. In PBL, the observed effect by this co-stimulation was probably attributable solely to IL-1β, as the overall response was similar to IL-1β treatment in PBL.

Afterwards, the putative association between PBMC surface Cp expression and oxidation of LDL was investigated. Thus, PBMN and PBL cultures were performed in medium

supplemented with native LDL and IL-1β. However, technical constraints hampered the detection of oxLDL levels in almost all samples. The only culture supernatants in which concentration of oxLDL was detected were the ones where PBMN were treated with IL-1β, but even so the average oxLDL concentration detected was low. In spite of these observations being consistent with the fact that surface Cp is increased in IL-1β treated PBMN, we cannot confirm at this time if GPI-Cp upregulation could be associated with oxidation of LDL, in a similar way as sCp [67]. In future experiments, a new incubation protocol should be optimized, in order to allow the measurement of sufficient levels of oxLDL in culture supernatants through ELISA technique *a posteriori*.

In order to complement this *in vitro* study, laboratorial characterization of FH patients, considered here as a clinical model of ATH, was performed. Also, the search of putative biochemical and immunological associations was attempted to further understand the interaction of Fe/Cu, lipid metabolism and inflammation at systemic level in this pathology. Comparisons between the control group and FH patients, showed that several parameters related to lipid metabolism (total cholesterol, LDL, triglycerides, ApoB and Lp(a)), were found significantly increased in the patients, which was consistent with levels normally found in individuals with a genetic defect in cholesterol metabolism such as FH, as previously reported [174]. Ferritin, oxLDL and IL-1β levels were increased in FH patients, but the difference was not statistically significant. Unexpectedly, sCp and CRP levels in FH patients did not differ from the ones in controls, but this may be accounted for the fact that at the time of blood collection most of these patients were being medicated by statins. It has been reported that this therapy is able to reduce CRP levels [175] decreasing the inflammation status of the individuals. Also, it must be taken into account the fact that most patients' serum samples were collected more than a year ago stored at -20ºC and, as such, some parameters might be influenced by time-dependent degradation in samples, particularly in the case of sCp. Moreover, several parameters were found to be correlated in FH patients and putatively associated with this ATH progression. sCp levels were positively correlated with CRP and oxLDL levels. These results are consistent with the fact both sCp and CRP are acute phase reactants and increased in inflammatory states [176], and the association between sCp and oxLDL gives further support to the reported pro-oxidant potential of sCp [67]. Furthermore, sCp was found to be negatively correlated with ferritin at systemic level, coherent with the ferroxidase role of sCp in Fe as it is negatively correlated with this storage protein and positively correlated with Tf (data not shown). Finally, in FH patients IL-1β levels were found to be positively correlated with total cholesterol and ApoB (the primary apolipoprotein of LDL), which are parameters commonly associated with increased CVD risk [177]. These observations were also in concordance with previous reports showing that individuals affected by dyslipidemia and/or chronic stable angina have higher circulating

levels of the proinflammatory/atherogenic cytokine IL-1β [105, 178], and that the lack of IL-1β decreases the severity of ATH [106]. However, the fact that no correlation was encountered between IL-1β and oxLDL or sCp does not allow us to conclude that the increased levels of oxLDL in this population might be a consequence of Cp expression induced by IL-1β, at least at systemic level. One should note that one limitation of this clinical study was the impossibility of this laboratorial characterization of FH patients being performed with fresh serum samples and before medication was administered. Taken into account this limitation, additional studies should be performed in order to further clarify the importance of these observations in atherosclerotic process and ATH outcome. Also, until the role of both isoforms of Cp is fully determined and the importance of GPI-Cp cell specific expression and regulation in putative pro-atherogenic conditions is addressed, the relevance of this protein in pathologies such as ATH and CVD cannot be completely elucidated. Nevertheless, the observations made during this study are an important and original contribution to clarify this issue.

Only through basic research one can start to understand the association between Fe/Cu status, lipid metabolism and inflammation in these processes. The fact that GPI-Cp was shown to be differently modulated by several of the putative pro-atherogenic treatments tested, and at times with opposite effects on PBMN compared to PBL, suggests the existence of specific cell-type regulation of this protein that might be associated with different physiological roles in each cell type. A shortcoming in this study was the ineffectiveness in the association of PBMC surface Cp expression with oxidation of LDL, but this should be easily addressed in future experiments. Of notice, the pro-atherogenic IL-1β which *in vitro* significantly induced GPI-Cp in PBMN population was found with increased levels in our clinical model of ATH. However the fact that no correlation was able to be observed between IL-1β and sCp or oxLDL suggest that, at least at systemic level, IL-1β may not be contributing factor in enabling oxidation of LDL by sCp.

In summary, the results of this study demonstrate that specific modulators of Fe/Cu status and inflammatory state putatively associated with ATH are involved in the modulation of Cp expression at surface of PBMC and thus, suggest that GPI-Cp could have a role in atherogenesis. Nevertheless, it is worth to mention that the complexity of ATH will not be explained only by the role of PBMC surface Cp and that the existence of other contributing factors and their interaction in plaque microenvironment should be fundamental pieces in the development of this disease, since *"nature doesn't recognize good or evil, only balance and imbalance"* [179].

FUTURE PERSPECTIVES

After treatment with several modulators the obtained results from *in vitro* experiments showed significant decreases of GPI-Cp expression associated with increased cellular activation. This type of expression might reveal a situation of protein shedding, which was not possible to evaluate during the course of this study. Thus, it would be important to analyze stored cultured supernatants and evaluate the presence of Cp by Western blot.

Several tested treatments were observed to be in contradiction with previous reports or with expected results. As such, the most promising treatments should be evaluated in different incubation conditions: IFN-γ, IL-2, IL-6, IL-8, M-CSF and TNF-α should be evaluated in shorter incubations periods to observe the immediate treatment effects on Cp expression, while reduced Cu status and SNAP treatment should be performed in longer incubations periods in order to evaluate the effect of protein stability in surface Cp expression levels. Due to time constraints it also proved to be difficult to test the effect of multiple modulators on GPI-Cp expression. As such, the evaluation of the effect of multiple pro-atherogenic stimuli co-incubated in cell cultures would be of particular interest, as to better mimic the *in vivo* plaque microenvironment.

One of the major limitations of this study was the failure to effectively evaluate the putative association between PBMC GPI-Cp expression and oxidation of LDL. The experimental protocol should be optimized to enable the incubation of native LDL in the appropriate conditions and the new designed experiments should also allow for the distinction of the secreted and membrane-bound Cp isoforms. In order to do so, after cellular stimulation with the selected treatment, supernatant and cell populations should be incubated separately in the presence of native LDL followed by the determination of oxLDL concentration induced by cells (GPI-Cp) or induced by supernatants (sCp).

In order to further complement these *in vitro* experiments, basal Cp expression levels of PBMC should be evaluated in the clinical model of FH patients. It would be necessary to perform a new patient recruitment, preferably with individuals before starting statin therapy. The objective of this characterization should allow to evaluate *in vivo* the effect of inflammatory state, Fe/Cu status and dyslipidemia associated with this clinical model and the pathology of ATH.

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Appendix I

Table S I - Significant correlations between biochemical parameters in total individuals (n=80)

Parameter	*versus*	r	*p* <
Total cholesterol	LDL	0,917	< 0,001
	Lp(a)	0,424	< 0,001
	ApoB	0,833	< 0,001
	Triglycerides	0,366	= 0,001
HDL	ApoA1	0,914	< 0,001
	Triglycerides	-0,317	< 0,01
	Transferrin	0,443	< 0,001
	Ferritin	-0,358	= 0,001
	Cp	375	= 0,001
LDL	Lp(a)	0,438	< 0,001
	ApoB	0,901	< 0,001
	Triglycerides	0,362	= 0,001
Lp(a)	ApoB	0,308	< 0,01
ApoA1	Transferrin	0,52	< 0,001
	Ferritin	-0,356	= 0,001
	Cp	0,501	< 0,001
ApoB	Triglycerides	0,507	< 0,001
Triglycerides	CRP	0,228	< 0,05
Iron	Ferritin	0,312	< 0,01
Transferrin	Ferritin	-0,529	< 0,001
	Cp	0,493	< 0,001
Ferritin	Cp	-0,408	< 0,001
CRP	Cp	0,535	< 0,001

Table S II - Significant correlations between biochemical parameters in FH patients (n=40)

Parameter	*versus*	r	*p*
Total cholesterol	LDL	0,975	< 0,001
	Lp(a)	0,438	< 0,01
	ApoB	0,838	< 0,001
	IL-1β	0,331	< 0,05
HDL	ApoA1	0,896	< 0,001
	Transferrin	0,348	< 0,05
LDL	Total cholesterol	0,975	< 0,001
	Lp(a)	0,470	< 0,01
	ApoB	0,861	< 0,001
Lp(a)	Total cholesterol	0,438	< 0,01
ApoA1	HDL	0,896	< 0,001
	Transferrin	0,326	< 0,05
ApoB	Total cholesterol	0,838	< 0,001
	Triglycerides	0,334	< 0,05
	IL-1β	0,345	< 0,05
Triglycerides	CRP	0,490	= 0,001
Iron	Ferritin	0,398	= 0,01
Transferrin	Ferritin	- 0.428	< 0,01
	CRP	- 0,344	< 0,05
Ferritin	sCp	- 0,359	< 0,05
CRP	sCp	0,375	< 0,05
sCp	oxLDL	0,523	= 0,001

Appendix II

Poster presented at European Iron Club Meeting 2011, 8th-11th September, Louvain-la-Neuve, Belgium.

INFLAMMATORY STIMULI MODULATES THE EXPRESSION OF CERULOPLASMIN AT SURFACE OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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INTRODUCTION

Atherosclerosis (ATH) is the leading cause of cardiovascular disease [1], and its development is characterized by the passage of LDL through the endothelial layer, and the early infiltration and activation of peripheral blood lymphocytes (PBL) and monocytes (PBMN) that contribute to a pro-inflammatory state in specific locations [2,3]. However, the functional interaction between immune cells and the oxidation of LDL is still not fully understood. One hypothesis for the etiopathogeny of ATH may be associated with an ongoing inflammatory process caused by a pro-oxidant/anti-oxidant imbalance induced by metals such as iron (Fe) or copper (Cu) [4]. Ceruloplasmin (Cp) is an acute-phase protein but also a multicopper oxidase with a relevant role in Fe metabolism mainly due to its ferroxidase activity [5], which is expressed in human PBL [6] and PBMN [7]. Herein, we aim to study the effect of putative pro-atherogenic immune stimuli on the expression of Cp at the surface of peripheral blood mononuclear cells (PBMC).

MATERIAL & METHODS

Isolation of Peripheral Blood Mononuclear Cells

Total peripheral blood from healthy volunteers was collected in citrate CPT™ vacutainer tubes (BD Biosciences) and PBMC were isolated according to the manufacturer's instructions. Remaining red blood cells (RBC) were removed by incubating PBMC with RBC lysis solution (10mM Tris, 16mM NH_4Cl, pH 7.4) for 10min at 37ºC, followed by washes in PBS before culture.

Immunomodulation of Cp expression

PBMC were incubated in RPMI 1640 at 37ºC in a 5% CO_2 atmosphere with/without the following stimulli: PMA (5 ng/mL, 2h), IL-1β (20, 50 ng/mL, overnight (o.n)), IL-6 (10, 50, 100 ng/mL, o.n), IL-8 (14, 73, 143 ng/mL, o.n) or TNF-α (2, 12, 20 ng/mL, o.n). PBMCs were then collected, ressuspended in Staining Buffer and subsequently analyzed by Flow Cytometry.

Immunophenotyping and Flow Cytometry analysis

Cells were plated in 96-well round-bottomed microtiter plates at 5×10^5 cells/well. PBMCs were stained for Cp by indirect staining using anti-human Cp (Koma Biotech) as primary antibody and goat F(ab')2 anti-rabbit FITC-conjugated (Rockland) as secondary antibody. Also, conjugated CD3-PE, CD69-PerCP, CD45-PerCP (BD Biosciences) and CD14-APC (MACS) antibodies were used to define PBL and PBMN populations under study. Flow cytometry analysis was performed in a FACScalibur (BD Biosciences) and protein expression levels were analyzed using Weasel v.2.7.4 software.

ANALYSIS OF RESULTS

PBMN SHOW HIGHER SURFACE CP EXPRESSION COMPARED TO PBL

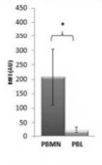

Fig.1 - Flow Cytometry analysis of surface Cp expression in PBMC. Surface Cp expression in untreated PBMN and PBL (n=16, *p < 0.01).

BOTH ACTIVATED PBMN AND PBL EXPRESS HIGHER SURFACE CP THAN THEIR NON-ACTIVATED COUNTERPARTS

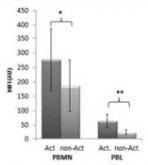

Fig.2 - Flow Cytometry analysis of surface Cp expression in PBMC. Surface Cp expression in untreated PBMN and PBL depending on their activation state (n=12, *p < 0.05, **p < 0.01).

T CELLS SHOW DECREASED SURFACE CP EXPRESSION COMPARED TO NON-T CELLS

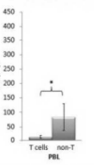

Fig.3 - Flow Cytometry analysis of surface Cp expression in PBL subpopulations. Surface Cp expression by in untreated PBL subpopulations (n=16, *p < 0.05).

CP EXPRESSION AT SURFACE OF PBMN AND PBL IS DIFFERENTLY MODULATED BY PMA AND IL-1β

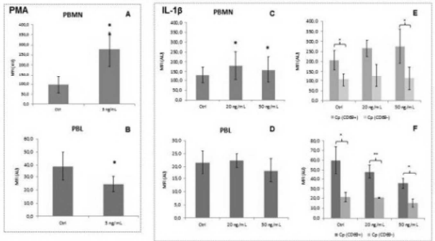

Fig.4 – Effect of PMA and IL-1β on surface Cp expression of PBMC. Results of flow cytometry analysis of surface Cp expression in untreated vs stimulated PBMN and PBL by PMA (A,B) and IL-1β (C-F). Analysis of Cp expression at surface of activated vs non-activated PBMN (E) and PBL (F) incubated with/without IL-1β. All experiments were performed in triplicate, *p < 0.05; **p < 0.01.

EXPRESSION OF CP AT SURFACE OF PBMC IS NOT MODULATED BY: IL-6, IL-8 AND TNF-α

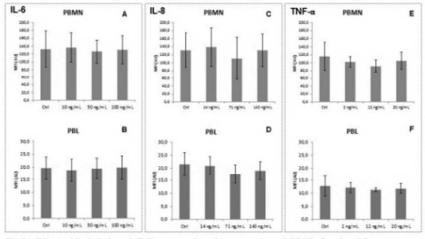

Fig.5 - Effect of IL-6, IL-8 and TNF-α on surface Cp expression of PBMC. Results of flow cytometry analysis of surface Cp expression in untreated versus stimulated PBMN and PBL by IL-6 (A,B), IL-8 (C,D) and TNF-α (E-F). All experiments were performed in triplicate.

CONCLUSIONS

The results obtained during this study showed:

- A consistently higher cell surface Cp expression in untreated PBMN *vs* PBL, activated *vs* non-activated cells and non-T *vs* T cells;
- Cp expression at surface of PBMN and PBL is differently modulated by PMA and IL-1β;
- Expression of Cp at surface of PBMC is not modulated by IL-6, IL-8 and TNF-α.

These results demonstrate that specific inflammatory mediators could be, at least in part, involved in the modulation of Cp expression at surface of PBMC and thus, could have a putative role in atherogenesis.

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