# LYMPHOCYTE CERULOPLASMIN AND BEHÇET'S DISEASE

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## ABSTRACT

**Introduction:** Behçet's disease (BD) is a rare chronic inflammatory disorder of unknown aetiology. However, it has been postulated that a dysregulation of the prooxidant/antioxidant balance may be important to its pathogenesis. Ceruloplasmin (CP) is an acute phase protein expressed at the surface of peripheral blood lymphocytes (PBL) with antioxidant properties and with a relevant role in iron (Fe) metabolism.
**Objectives**: To study CP expression at the surface of PBL (PBLCP) in patients with BD.
**Material and Methods**: We measured serum CP and PBLCP obtained from BD patients (n=10) and respective controls (n=10) using nephelometry and flow cytometry techniques, respectively. Additionally, haematological parameters, biochemical Fe metabolism markers [serum Fe, serum ferritin, serum transferrin, total Fe binding capacity (TIBC), transferrin saturation] and non-specific markers of inflammation [serum C reactive protein (CRP), $\beta_2$-microglobulin] were measured in all individuals.
**Results**: Despite the absence of significant differences between the two study groups when comparing serum CP, a significant difference in PBLCP was found in BD patients mainly due to a significant decrease of CP expression at the surface of $CD3^-CD56^+$ lymphocytes. Also, a significant decrease of PBLCP was observed in patients treated with azathioprine compared to patients that were not being treated with this drug.
**Conclusions**: According to this study, we suggest that the significant decrease of PBLCP observed in BD patients might be due to azathioprine treatment and not directly related to the pathophysiology of BD.

**Key-Words**: Behçet's disease; Ceruloplasmin; Prooxidant/antioxidant Balance; Lymphocytes; Azathioprine.

## RESUMO

**Introdução**: A Doença de Behçet (DB) é uma doença crónica rara de etiologia desconhecida. Estudos anteriores mostram que um desequilíbrio no balanço pró-oxidante/antioxidante é importante para a sua patogénese. Por outro lado, a ceruloplasmina (CP) é uma proteína de fase aguda expressa à superfície de linfócitos do sangue periférico de humanos (CPL), com actividade antioxidante e com um papel relevante no metabolismo do ferro (Fe).
**Objectivos**: Estudo da expressão da CPL em indivíduos com DB.
**Material e Métodos**: Mediu-se a CP sérica e a CPL em doentes com DB (n=10) e controlos (n=10), utilizando técnicas de nefelometria e citometria de fluxo, respectivamente. Adicionalmente, determinaram-se parâmetros hematológicos, marcadores bioquímicos séricos do metabolismo do Fe [Fe, ferritina, transferrina, capacidade total de fixação do Fe, saturação de transferrina] e marcadores inespecíficos de inflamação [proteína C reactiva (PCR) e $\beta_2$-microglobulina] em todos os indivíduos.
**Resultados**: Apesar de não existirem diferenças significativas entre os dois grupos relativamente à CP sérica, observou-se nos doentes uma diferença significativa na CPL devido essencialmente ao decréscimo significativo da expressão da CP à superfície dos linfócitos $CD3^-CD56^+$. Contudo, a análise comparativa da CPL entre indivíduos com DB medicados com azatioprina e não medicados, mostrou a existência de uma diminuição significativa da CPL nos indivíduos tratados com este medicamento.
**Conclusões**: Os resultados deste estudo sugerem que o decréscimo da CPL verificado nos doentes com DB está associada a um efeito da medicação e não à fisiopatologia desta doença.

**Palavras-chave**: Doença de Behçet; Ceruloplasmina; Balanço pró-oxidante/antioxidante; Linfócitos; Azatioprina.

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## Introduction

Behçet's disease (BD) is a rare chronic vasculitis of unclear aetiology.[1] BD is characterized by recurrent oral and genital ulcers, skin and ocular lesions (uveitis and retinal vasculitis). Other manifestations include arthritis, thrombophlebitis, arterial lesions (arterial occlusion and aneurysm), central nervous system (CNS) involvement and gastrointestinal ulcerations.[2]

Since there is no specific laboratory marker for BD, its diagnosis still remains clinical. Therefore, the diagnostic criteria proposed by the «International Study Group of Behçet's Disease» (ISGBD) in 1990 are widely accepted.[3] According to these criteria BD patients are characterized as having recurrent oral ulcers and, at least, two of the following symptoms: recurrent genital ulcers, eye lesions, skin lesions or positive pathergy test.

Although the aetiology of BD remains unknown, several reports suggest an excessive production of reactive oxygen species (ROS) by activated neutrophils. Also, a diminished enzymatic antioxidant activity might have a crucial role in tissue lesions characteristic of this disease.[4,5] Particularly, it has been described in BD patients an increase in the plasma levels of some oxidative stress markers such as nitric oxide (NO), nitrite, nitrate[6] and malondialdehyde (MDA),[7] a final product of the lipid peroxidation from ROS action. Other reports showed a decrease in plasma glutathione peroxidase (GSH-Px) activity[7] as well as in the activity of reduced glutathione (GSH),[8] suggesting a decrease of the antioxidant activity in these patients. Additionally, dysregulation in lipid metabolism has been reported in BD patients including the increase of lipoproteins, lipid hydroperoxides and susceptibility to oxidation of low density lipoproteins (LDL)[9] associated with a decrease of paraoxonase (PON) activity, indicating the development of an endothelial dysfunction.

Ceruloplasmin (CP) is an abundant plasma $\alpha_2$-glicoprotein included in a family of proteins called multicopper oxidase enzymes.[10] CP contains three types of spectroscopically distinct copper (Cu) sites which incorporate 6 or 7 Cu atoms during CP biosynthesis. Although the liver is the predominant source of serum CP, extrahepatic CP gene expression has been demonstrated in many tissues including lungs, testicles and CNS.[11] Recently preliminary studies by our group showed that peripheral blood lymphocytes (PBL) are able to synthesize CP,[12] expressing it at their surface[13-16] and releasing it into the extracellular milieu.[17]

CP possesses an antioxidant role due to its ferroxidase activity in iron (Fe) metabolism.[11] The oxidation of ferrous ion ($Fe^{2+}$) to ferric ion ($Fe^{3+}$) by CP is thought to reduce oxidative stress by preventing the Fenton reaction in which $Fe^{2+}$ generates ROS. This reaction is also crucial for the Fe mobilisation and release from cellular stores for uptake by the circulating iron transport protein transferrin (Tf).[18]

It has been shown that humans with mutations in the CP gene (aceruloplasminemia patients) have Fe accumulation in the brain leading to neurodegeneration.[19] Also, CP knockout mice (CP^{-/-}) show excessive Fe content in the brain with ageing.[20]

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Moreover, CP may act as an acute phase reactant being involved in protective cellular mechanisms mediated by the immune system (IS). Accordingly, CP levels are increased in several clinical settings including infection, trauma and inflammation.[10]

An increase in serum CP was previously reported in BD patients.[4,7] However, to our knowledge the expression of CP at surface of PBL has never been studied in these patients.

## Objectives

The main goal of this work was to study CP expression at the surface of PBL in patients with BD.

## Material and Methods

### Individuals

Ten BD patients, that fulfilled the international criteria proposed by the ISGBD, of both genders, with more than 20 and less than 60 years old, were selected from the Instituto Português de Reumatologia (IPR) outpatient clinic for this study (BD group). All the assays were performed in 10 healthy blood donors age-gender matched, recruited from the Hospital Reynaldo dos Santos (HRS) [control (C) group].

Disease duration was 1-29 years (11,7 ± 2,7 years, mean ± standard error). At the time of blood collection, 50% of the patients were considered active and 50% inactive according to the clinical evaluation of the 4 previous weeks.

Of the ten patients, eight were receiving treatment, namely oral colchicine (30% of the BD group), azathioprine (50% of the BD group) and/or corticosteroids (30% of the BD group). The three patients receiving colchicine had the last intake 48h before blood collection, while in the five patients receiving azathioprine only one took it in the same day of the blood collection. The other four patients that were on azathioprine treatment were medicated more than 12h before sample collection. Also, corticosteroids had been administered more than 24h before blood collection.

All the patients in the study had typical involvement including oral (90%) and genital (60%) aphta, uveitis (40%), skin lesions (70%) and arthritis (40%).

Collection of total peripheral blood was done after all participants had given their informed consent.

### Haematological and biochemical measurements

Cell blood count were performed in EDTA collected peripheral blood from volunteers using an automated haematology counter Coulter MAXM and included: measurement of haemoglobin concentration (Hb), red cell count (RBC), packed cell volume (PCV), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red cell distribution width (RDW), white cell count (WBC) and differential white cell count.

Serum Fe and Tf were measured using an automated analyser BM/Hitachi 911 (Boehringer Mannheim, Roche) by a coloured enzymatic test. Tf saturation was calculated from the total iron binding capacity (TIBC) and serum Fe values. Quantitative measurements of serum ferritin (Ft) were performed by an immunometric assay using an Immulite analyser. Serum CP, C reactive protein (CRP) and $\beta_2$-microglobulin ($\beta_2$m) were measured by nephelometry using a Beckman Immage analyzer.

### Flow cytometry analysis of surface expression of ceruloplasmin in peripheral blood lymphocytes

Fresh peripheral blood cells were obtained from 1 ml of EDTA collected peripheral blood. RBC were lysed in lysis solution (10mM Tris, 16mM $NH_4Cl$, pH 7.4) for 10 min at 37°C and the remaining white blood cells were then washed in PBS supplemented with 0.2% of bovine serum albumin (BSA), resuspended and plated in round-bottomed microtitre plates (Nunclon, Denmark) at $3 \times 10^5$ cells/well.

Cells were stained for CP using the rabbit anti--human CP (DakoCytomation, Denmark) as primary antibody (Ab) followed by incubation with a swine $F(ab')_2$ anti-rabbit FITC-conjugated as secondary Ab (DakoCytomation, Denmark). To determine CP's mean fluorescence intensity (MFI) in specific lymphocyte subsets, monoclonal Ab (mAb) CD4-PE, CD8-PE, CD19-PE or CD56-PE conjugated were used separately combined with mAb CD45-PerCP conjugated for lymphocyte gating and mAb CD3-APC conjugated for positive or negative selection of T cells. Non-stained cells were used as negative control, to determine autofluorescence. All mAb were purchased from Pharmingen.

After staining, cells were washed twice in PBS//BSA solution, resuspended in FACS Flow solution and flow cytometry analysis was performed using a FACSCalibur (Becton&Dickinson). Analysis of data was done using the CellQuest™ Software.

Results are presented in Arbitrary Units (AU) resulting from the ratio between the MFI of stained cells and the MFI of non-stained cells in the same population.

## Statistical Analysis

All results were given as mean ± standard error (SE). Statistical analysis was performed using the Mann-Whitney and Kruskall-Wallis tests. Multiple comparisons between groups were performed using the Dunnett T3 Test. Values of $p \leq 0,05$ were accepted as statiscally significant. SPSS Base 14.0 software was used to perform all statistical analysis (SPSS inc. 2005).

## Results

In this work, 10 patients with BD [7 women (70%) and 3 men (30%)] and a control (C) group formed by 10 healthy blood donors [7 women (70%) and 3 men (30%)] were studied. The age for BD group was 42,7 ± 3,1 years old with a minimum of 23 and a maximum of 55 years old, while for the C group the age was 44,1 ± 3,5 years, in a range from 26 to 60 years old.

Results obtained concerning the measurement of haematological parameters showed no significant differences between BD and C groups.

Despite the absence of significant differences in serum Fe and Ft concentrations measured in both groups, significant increases ($p$=0,014) in serum Tf (298,7 ± 13,3 mg/dL $vs$ 251,5 ± 10,7mg/dL; p=0,014)

and TIBC (373,4 ± 16,6mg/dL $vs$ 314,4 ± 13,4mg/dL) were observed in BD patients compared to controls (Table I). However, no significant difference was found between BD patients and controls when comparing concentration of CP, CRP and $\beta_2$m in serum (Table I).

Additionally, results from flow cytometry analysis (Figure 1) showed a significant decrease of PBLCP from BD patients comparatively to controls (48,4±7,7 AU $vs$ 77,7±10,9 AU; $p$=0,041). This difference was mainly due to a significant decrease ($p$=0,041) of CP surface expression in $CD3^-CD56^+$ cells (NK cells) of BD patients when compared to $CD3^-CD56^+$ cells from controls (164,3±37,5 AU $vs$ 323,62±57,6 AU; Figure 2).

In order to understand these results, an additional comparison between PBLCP from BD patients under treatment and PBLCP from controls was performed. No significant differences were found between PBLCP from patients treated with colchicine (and/or corticosteroids) and PBLCP from controls (data not shown). However, BD patients treated with azathioprine showed a significant decrease of PBLCP ($p$=0,005) compared to controls (29,7 ± 3,7 AU $vs$ 77,7 ± 10,9, respectively; Figure 3). Also, a significant decrease of PBLCP ($p$=0,026) was found when comparing patients treated with azathioprine with non-azathioprine treated patients (29,7 ± 3,7 AU $vs$ 67,2 ±8,8 AU, respectively; Figure 3). Moreover, no significant difference in PBLCP was found between non-azathioprine treated patients and controls (67,2 ±8,8 AU $vs$ 77,7 ± 10,9, respectively; Figure 3).

### Table I. Iron metabolism and inflammatory proteins measured in serum from BD patients (BD group) and controls (C group).

| Iron Metabolism Parameters | C group (Mean±SE) | BD group (Mean±SE) |
|---|---|---|
| Fe (μg/dL) | 103,2 ± 5,7 | 105,3 ± 10,3 |
| Ft (ng/dL) | 68,6 ± 22,9 | 119,5 ± 25,1 |
| Tf (mg/dL) * | 251,5 ± 10,7 | 298,7 ± 13,3 |
| TIBC (mg/dL) * | 314,4 ± 13,4 | 373,4 ± 16,6 |
| **Inflammatory proteins** | | |
| CP (mg/dL) | 31,4 ± 2,2 | 39,9 ± 4,6 |
| CRP (mg/dL) | 0,3 ± 0,1 | 0,5 ± 0,1 |
| $\beta_2$-m (μg/L) | 983,2 ± 68,3 | 1068,8 ± 64,0 |

Fe – iron; Ft – ferritin; Tf – transferrin; TIBC – total iron binding capacity; CP – serum Ceruloplasmin; CRP – C Reactive Protein; $\beta_2$m – $\beta_2$-microglobulin; *: p=0,014.

## Discussion

Oxidative stress has been implicated in the pathogenesis of certain disorders including BD.[21] Disturbance in the prooxidant/antioxidant balance and the production of free radicals play a significant role in this process.[4] On the other hand, Fe metabolism and oxidative stress are clearly interactive, especially under pathological conditions. In fact, Fe is extremely important due to its capacity of acting both as a donor and acceptor of electrons.[22] However, this property allows Fe to be potentially toxic since it catalyses the conversion of hydrogen peroxide ($H_2O_2$) to free radicals. Proteins such as Tf
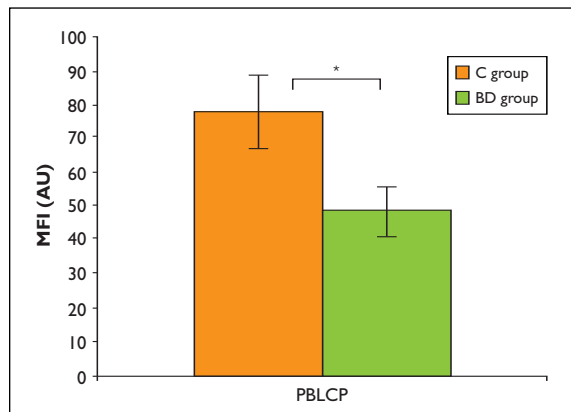
**Figure 1.** Mean fluorescence intensity (MFI) ratio of CP at the surface of peripheral blood lymphocytes (PBLCP) in BD patients (BD group) and controls (C group) (*: p= 0,041).
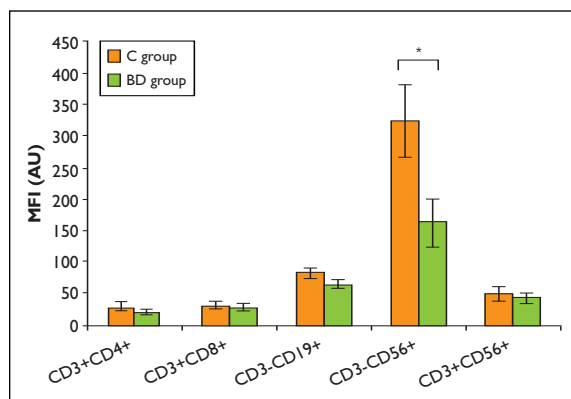


**Figure 2.** Mean fluorescence intensity (MFI) ratio of CP at the surface of lymphocyte subpopulations in BD patients (BD group) and controls (C group). CD3$^+$CD4$^+$ = T helper lymphocytes; CD3$^+$CD8$^+$ = T cytotoxic lymphocytes; CD3$^-$CD19$^+$ = B lymphocytes; CD3$^-$CD56$^+$ = Natural Killer (NK) lymphocytes; CD3$^+$CD56$^+$ = NKT lymphocytes (*: p= 0,41).

and Ft help to prevent this oxidative attack by sequestering Fe and avoiding radicals formation and/or lipid peroxidation.[23]

In this study, an increase in serum Fe and Ft concentrations and a significant increase of serum levels of Tf and TIBC were observed in the BD group comparatively to controls (Table I). These results suggest that the raise of Tf and Ft observed in BD patients may be related to a possible compensatory mechanism to limit oxidative stress in this disease.

During the active phase of the disease, BD is associated with many non-specific features of systemic inflammation, including an increase in the le-
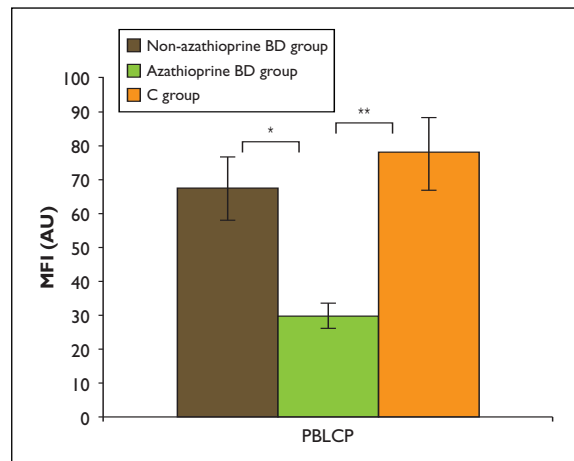


**Figure 3.** Mean fluorescence intensity (MFI) ratio of CP at the surface of peripheral blood lymphocytes (PBLCP) in BD patients treated with azathioprine (azathioprine BD group), non-azathioprine treated BD patients (non-azathioprine BD group) and control group (C group) (*: p= 0,026; **: p= 0,005).

vel of circulating pro-inflammatory cytokines, increased CRP and $\beta_2$m.[24] The results obtained in this study give additional support for these previous findings, since an increase of CRP and $\beta_2$m, although not significant, was found in BD patients comparatively to controls. On the other hand, serum CP is also considered an acute phase reactant. Accordingly, an increase of CP serum concentrations has already been reported in BD patients in other studies.[25] Although not statistically significant, results obtained in this study also showed an increase of serum CP levels in BD group compared to controls. However, a significant decrease in PBLCP was found in BD patients comparatively to controls. This decrease seemed to be due to a significant reduction of PBLCP observed in BD patients treated with azathioprine. In fact, these patients showed a significant decrease of PBLCP when compared to non-azathioprine treated BD patients. Furthermore, a decrease in PBLCP was also found when comparing azathioprine treated patients with the respective controls. Overall, these results suggest that azathioprine treatment might have a direct effect on CP expression at the cell surface of PBL.

## Conclusions

The results obtained in this study suggest that the

significant decrease of PBLCP found in BD patients comparatively to controls is due to the azathioprine treatment and not related to BD. However, further studies including a larger number of patients are needed to fully understand the role played by PBLCP in BD pathophysiology.

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