

Original Contribution

# Ceruloplasmin expression by human peripheral blood lymphocytes: A new link between immunity and iron metabolism

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## Abstract

Ceruloplasmin (CP) is a multicopper oxidase involved in the acute phase reaction to stress. Although the physiological role of CP is uncertain, its role in iron (Fe) homeostasis and protection against free radical-initiated cell injury has been widely documented. Previous studies showed the existence of two molecular isoforms of CP: secreted CP (sCP) and a membrane glycosylphosphatidylinositol (GPI)-anchored form of CP (GPI-CP). sCP is produced mainly by the liver and is abundant in human serum whereas GPI-CP is expressed in mammalian astrocytes, rat leptomeningeal cells, and Sertoli cells. Herein, we show using RT-PCR that human peripheral blood lymphocytes (huPBL) constitutively express the transcripts for both CP molecular isoforms previously reported. Also, expression of CP in huPBL is demonstrated by immunofluorescence with confocal microscopy and flow cytometry analysis using cells isolated from healthy blood donors with normal Fe status. Importantly, the results obtained show that natural killer cells have a significantly higher CP expression compared to all other major lymphocyte subsets. In this context, the involvement of lymphocyte-derived CP on host defense processes via its anti/prooxidant properties is proposed, giving further support for a close functional interaction between the immune system and the Fe metabolism.

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**Keywords:** Ceruloplasmin; Peripheral blood lymphocytes; Iron; Copper; NK cells

## Introduction

The molecular link between iron (Fe) and copper (Cu) metabolism has been identified as the serum multicopper protein

*Abbreviations:* CP, ceruloplasmin; CTL, cytotoxic T lymphocyte; Cu, copper; Fe, iron; Ft, ferritin; GPI-CP, membrane glycosylphosphatidylinositol-anchored ceruloplasmin; HBSS, Hank's balanced salt solution; HH, hereditary hemochromatosis; huPBL, human peripheral blood lymphocytes; huPBMn, human peripheral blood monocytes; LDLR, low-density lipoprotein receptor; mAb, monoclonal antibody; MHC, major histocompatibility complex; NK cell, natural killer cell; NKT cell, natural killer T cell; PBMC, peripheral blood mononuclear cells; RT-PCR, reverse transcription-polymerase chain reaction; sCP, secreted ceruloplasmin; Tf, transferrin; Th, T helper (cell).

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ceruloplasmin (CP) [1]. CP is an abundant  $\alpha_2$ -glycoprotein containing over 95% of the Cu found in the plasma of all vertebrate species [2]. The protein is synthesized mainly in the liver as a single polypeptide chain of 1046 amino acids and secreted into the plasma with six to seven atoms of Cu bound per molecule. CP has been implicated in Fe metabolism mostly because of its catalytic oxidation of Fe(II) to Fe(III) (ferroxidase activity) [3] with its subsequent incorporation into apotransferrin [4] or into the Fe storage protein ferritin (Ft) [5]. Nevertheless, the physiological role of CP is not well defined but may include extracellular antioxidant activity by promoting Fe mobilization and thus preventing metal-catalyzed free radical tissue damage [6,7]. Alternatively, several studies suggest that CP may also exhibit potent prooxidant activity [8]. Despite the unknown function of such CP prooxidative activity, it is likely that the protein is involved in host defense and repair processes mediated by the immune system, namely

during inflammation [9] and hyperoxia [10]. In fact, CP is an acute-phase reactant with its serum concentration increasing during pregnancy, infection, tissue injury, and certain malignant disorders [11]. Supporting the possible role of CP in host protective function, early studies have shown that respiratory burst and microbicidal activity of phagocytic cells are suppressed by Cu deficiency [12,13] while changes in splenic lymphoid cell subsets have been observed in Cu-deficient rats [14]. In addition, several cytokines and other factors are known to induce CP synthesis by hepatic cells including interferon-gamma [15], interleukin-1, interleukin-6 [16], tumor necrosis factor alpha, and lipopolysaccharide [17], suggesting a link between this protein and immune function.

On the other hand, the link between Fe metabolism and cell-mediated immunity is now well established [18]. The postulate that the immune system could have a role in monitoring tissue Fe toxicity as part of its surveillance function was reported over 25 years ago [19]. Immunological anomalies observed in patients with hereditary hemochromatosis (HH) [20–22] and animal models of Fe overload [23] strongly supported this original concept. Also, the discovery of the HFE gene associated with HH as a new major histocompatibility complex (MHC) class I gene [24] provided conclusive evidence for the role of the immune system regulating Fe homeostasis. However, a direct relationship among human peripheral blood lymphocytes (huPBL), CP, and Fe metabolism has never been investigated.

Although CP is generally considered a serum protein secreted by the liver, extrahepatic expression has also been observed [25–27]. In particular, a membrane-bound glycosylphosphatidylinositol (GPI)-anchored form of CP (GPI-CP) localized at the surface of mammalian astrocytes [28], rat leptomeningeal cells [29], and Sertoli cells [30] was reported. However, despite the detection of CP mRNA in immune cells [31,32], the characterization of the specific molecular isoform (s) expressed by huPBL has never been attempted.

In this study, reverse transcription-PCR (RT-PCR) was used to examine whether huPBL constitutively express CP and to identify its specific transcripts. Also, the expression of the mature CP protein in the major lymphocyte subpopulations isolated from peripheral blood collected from healthy volunteers with normal iron status was investigated by flow cytometry while protein localization was studied by confocal microscopy analysis of immunofluorescence staining. The results obtained showed that huPBL express both secreted CP (sCP) and GPI-CP transcripts. Also, we showed that CP protein is localized at the huPBL surface. Furthermore, we demonstrated that CP expression at the huPBL surface is highly specific to the natural killer (NK) cell lymphocyte subset, suggesting an important role for huPBL-associated CP in the relationship among innate immunity, Fe metabolism, and oxidative stress.

## Materials and methods

### Individuals

Twenty-four healthy volunteers from both genders (11 women and 13 men) aged 20 to 60 years ( $40.9 \pm 2.22$  years,

mean age  $\pm$  SD) were included in this study (INSA, Lisbon). After informed consent, peripheral blood samples were obtained by venous puncture to determine hematological and biochemical parameters of Fe metabolism. Exclusion criteria included any Fe loading or deficiency controlled by measurement of hematological and biochemical Fe metabolism markers. The study was approved by the INSA-Ethical Committee.

### Hematological and biochemical markers of Fe metabolism

Cell blood counts were performed in EDTA-collected peripheral blood from healthy human volunteers using an automated hematology counter Coulter MAXM and included determination of hemoglobin (Hb), red blood cell (RBC) count, mean cell volume (MCV), mean cell Hb (MCH), mean cell Hb concentration (MCHC), white blood cell (WBC) count, and differential WBC count.

Serum Fe, transferrin (Tf), and total Fe binding capacity (TIBC) were measured using an automated analyzer BM/Hitachi 911 (Boehringer Mannheim, Roche) by an enzymatic colorimetric assay. Tf saturation was calculated from the TIBC and serum Fe values. Quantification of serum Tf was performed by an immunometric assay using an Immulite analyzer. Serum CP was measured by nephelometry using a Beckman Array System analyzer.

### Cell isolation and preparation for RNA extraction

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by density gradient centrifugation using Lymphoprep (Axis-Shield, Norway) and washed with Hank's balanced salt solution (HBSS) (GIBCO BRL, Invitrogen). RBC were lysed in lysis solution (10 mM Tris, 16 mM  $\text{NH}_4\text{Cl}$ , pH 7.4) for 10 min at 37 °C. The remaining PBMC were washed in HBSS and resuspended in RPMI 1640 culture medium supplemented with GlutaMAX I, 25 mM Hepes buffer (GIBCO BRL, Invitrogen), and 10% fetal bovine serum (GIBCO BRL, Invitrogen). For lymphocyte enrichment, each PBMC suspension was seeded in T75 culture flasks followed by a 2-h incubation at 37 °C in an atmosphere of 5%  $\text{CO}_2$ . Cells in suspension were collected and washed in HBSS. For monocyte-enriched PBMC, the remaining adherent cells in the culture flasks were washed with HBSS several times until no cells were observed in suspension. Cells were then collected and washed in HBSS.

HepG2 cells cultured in William's E medium (GIBCO BRL, Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (GIBCO BRL, Invitrogen) were collected from a confluent T25 culture flask and washed in Dulbecco's PBS (GIBCO BRL, Invitrogen).

### RNA extraction and RT-PCR

Human lymphocyte-enriched PBMC, monocyte-enriched PBMC, and HepG2 cells were lysed in RLT buffer (QIAGEN) with 1%  $\beta$ -mercaptoethanol, homogenized by passing the lysates through a 20 G needle for at least 10 times followed by

centrifugation in a QIAshredder spin column (QIAGEN). The homogenized lysates were stored at  $-80^{\circ}\text{C}$  until RNA extraction. Total RNA was extracted according to RNeasy Protect Mini Kit protocol (QIAGEN), followed by quantitative and qualitative analysis. cDNA was synthesized with oligo(dT) primers according to the Superscript First-Strand cDNA Synthesis System procedure (Invitrogen). The resulting cDNA strand was then used as template for PCR with primers specifically designed for low-density lipoprotein receptor (LDLR) [33], CD81 (lymphocyte-specific marker), CD33 (monocyte and macrophage-specific marker), sCP, and GPI-CP. Primers were designed for the 3' terminal sequence of CP transcripts in order to distinguish between sCP and GPI-CP mRNA, with the reverse primers being designed for exons 19 and 20, respectively. The sequences of all primers are shown in Table 2 (view Supplementary data). The PCR protocol was as follows: each reaction contained 0.2 mM of each deoxynucleotide triphosphate (dNTP) (Bioline, UK), 1.5 mM  $\text{MgCl}_2$  (Bioline, UK) for LDLR, CD81, and CD33 PCR and 3.0 mM  $\text{MgCl}_2$  for sCP and GPI-CP PCR conditions, 10 pmol of each primer (Invitrogen), 1.0 units of Biotaq polymerase (Bioline, UK), 10X ammonium buffer (Bioline, UK), 1–3  $\mu\text{l}$  of each cDNA sample (100–200 ng/ $\mu\text{l}$ ), and water up to a final volume of 25  $\mu\text{l}$ . For CD33 and GPI-CP PCR, 10% dimethyl sulfoxide was also used. The PCR conditions are shown in Table 3 (view Supplementary data). After final extension, all RT-PCR products were cooled down and stored at  $4^{\circ}\text{C}$  until posterior analysis.

RT-PCR products were separated in a 1.5% agarose gel (GIBCO BRL Life Technologies, Invitrogen) stained with ethidium bromide (Sigma–Aldrich) and analyzed using Quantity One 4.3.0 software (Bio-Rad Laboratories). Amplified fragments were purified using EXOSAP (GE Healthcare, Sweden), followed by DNA sequencing. The resulting sequences were analyzed using BioEdit 7.0.5.3 software [34] for confirmation of RT-PCR products identity.

#### Immunophenotyping and flow cytometry analysis

Fresh peripheral blood cells were obtained from 1 ml of EDTA-collected peripheral blood. RBC were lysed in lysis solution for 10 min at  $37^{\circ}\text{C}$  and the remaining white blood cells were then washed in PBS supplemented with 0.2% BSA (Sigma–Aldrich), resuspended, and plated in 96-well round-bottomed microtiter 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')<sub>2</sub> anti-rabbit FITC-conjugated as secondary Ab (DakoCytomation, Denmark). To determine CP mean fluorescence intensity (MFI) in specific lymphocyte subsets, monoclonal Ab (mAb) CD4-PE, CD8-PE, CD19-PE, or CD16/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. HuPBL subpopulations within the “lymphogate” were designated according to the expression of its surface markers: T helper lymphocyte (Th) (CD3+CD4+), cytotoxic T lymphocyte (CTL) (CD3+CD8+), B lymphocytes (CD3-CD19+), NK (CD3-CD16+/CD56+), and natural killer T

(NKT) cells (CD3+CD16+/CD56+). Absence of unspecific binding of anti-rabbit FITC-conjugated secondary Ab was verified by cell staining in the absence of primary Ab. Also, unstained 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 and resuspended in FACS Flow solution followed by flow cytometry analysis using a FACSCalibur (Becton Dickinson). Analysis of data was performed 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.

#### Immunofluorescence staining for confocal microscopy analysis

Lymphocyte-enriched human PBMC samples were prepared as described previously. Cells were washed, fixed in 4% formaldehyde in PBS at  $4^{\circ}\text{C}$  for 30 min, washed, and preserved in PBS at  $4^{\circ}\text{C}$ . Cells were adhered onto silane-coated slides (Sigma–Aldrich) using Shandon cytospin III (GPI, Inc., USA) and blocked with 1% BSA in PBS for 45 min at room temperature, followed by a 45-min incubation with 1:50 sheep anti-human CP IgG-FITC conjugated (BIOTREND GmbH, Germany) diluted in blocking solution at room temperature. Cells were washed three times with 0.5% BSA in PBS for 5 min at room temperature. Dyes were mounted in Vectashield conjugated with DAPI (Vector laboratories, Ltd., UK) and observed by confocal microscopy.

#### Confocal microscopy analysis

Dyes were imaged using a Bio-Rad MCR-600 (Microscience Ltd, Hemel Hempstead, UK) confocal laser scanning microscope

Table 1

Hematological and biochemical markers of Fe metabolism measured in peripheral blood obtained from human healthy volunteers ( $n=24$ , mean $\pm$ SE)

	Total ( $n=24$ )	Men ( $n=11$ )	Women ( $n=13$ )	P <sup>b</sup>
Hb (g/dl)	14,3 $\pm$ 0,2 <sup>a</sup>	15,3 $\pm$ 0,2	13,6 $\pm$ 0,3	0,013
RBC ( $\times 10^{12}/\text{l}$ )	4,7 $\pm$ 0,8	4,9 $\pm$ 0,1	4,5 $\pm$ 0,1	0,034
MCV (fl)	87,3 $\pm$ 0,9	86,0 $\pm$ 1,3	89,0 $\pm$ 1,9	n.s.
MCH (pg)	30,6 $\pm$ 0,3	30,9 $\pm$ 0,5	30,2 $\pm$ 0,8	n.s.
MCHC (g/dl)	35,1 $\pm$ 0,3	33,3 $\pm$ 0,53	34,08 $\pm$ 0,56	0,000
WBC ( $\times 10^6/\text{ml}$ )	6,3 $\pm$ 0,4	6,1 $\pm$ 0,5	6,6 $\pm$ 0,9	n.s.
Neutrophils ( $\times 10^6/\text{ml}$ )	3,6 $\pm$ 0,2	3,8 $\pm$ 0,3	3,5 $\pm$ 0,3	n.s.
Monocytes ( $\times 10^6/\text{ml}$ )	0,5 $\pm$ 0,1	0,5 $\pm$ 0,1	0,4 $\pm$ 0,1	n.s.
Lymphocytes ( $\times 10^6/\text{ml}$ )	2,0 $\pm$ 0,2	1,6 $\pm$ 0,2	2,4 $\pm$ 0,6	n.s.
Fe ( $\mu\text{g}/\text{dl}$ )	99,6 $\pm$ 5,7	85,8 $\pm$ 11,5	96,8 $\pm$ 8,4	n.s.
Tf (mg/dl)	252,5 $\pm$ 8,3	250,2 $\pm$ 15,0	262,0 $\pm$ 15,1	n.s.
TIBC (mg/dl)	315,6 $\pm$ 10,4	313,0 $\pm$ 18,9	327,5 $\pm$ 18,8	n.s.
Tfsat (%)	32,0 $\pm$ 2,0	27,8 $\pm$ 4,2	30,1 $\pm$ 3,1	n.s.
Ft (ng/ml)	84,2 $\pm$ 16,3	130,7 $\pm$ 39,6	39,8 $\pm$ 12,0	0,018
CP (ng/ml)	30,2 $\pm$ 1,5	27,9 $\pm$ 5,85	35,3 $\pm$ 6,66	0,009

n.s., not significant. Hemoglobin, Hb; red blood cell count, RBC; mean cell volume, MCV; mean cell hemoglobin, MCH; mean cell hemoglobin concentration, MCHC; white blood cell count, WBC; serum iron, Fe; transferrin, Tf; total iron binding capacity, TIBC; Tf saturation, Tfsat; serum ferritin, Ft; serum ceruloplasmin, CP.

<sup>a</sup>Arithmetic mean $\pm$ 1 standard error.

<sup>b</sup>Comparison between genders (men and women).

(CLSM) coupled to an Olympus BX-51 scope. Images were collected in F1 scanning mode (~1 s per frame), with a 3% laser intensity, an electronic zoom of  $\times 2$  or  $\times 3$ . A  $\times 60$  Plan Apo dry objective (NA=0.85) (Olympus) was used. The black level and gain settings were adjusted so that the average background pixel intensity was between 0 and 10 and the fluorescent signal coming from the cells was between 40 and 220. The degree of filter block cross talk was estimated and a simple method was devised to establish the confocal settings at which cross talk was minimal [35]. Once defined, the settings were kept constant throughout the data collection. Fluorescence analysis was performed with the software package Image-Pro Plus 4.0 (Media Cybernetics).

#### Statistical analysis

The association between CP expression in total huPBL and CP expressed in differential lymphocyte subpopulations (Th, CTL, B, NK, NKT cells) was measured by the Pearson and Spearman correlation coefficients. Confidence intervals for mean at 95% level were calculated.

In order to estimate the impact between CP expression in total huPBL and CP expressed in each lymphocyte subsets, a linear regression model was adjusted ( $Y = \alpha + \beta x + \varepsilon$ , where  $Y$  is

the dependent variable,  $x$  is the independent variable,  $\alpha$  is the constant,  $\beta$  is the coefficient of independent variable, and  $\varepsilon$  is the random error).

For model diagnosis we used the adjusted  $R^2$ , Kolmogorov–Smirnov test for the assumption “Normally” distributed residuals and the Durbin–Watson for the assumption of autocorrelation. Values of  $P \leq 0.05$  were accepted as statistically significant. SPSS Base 14.0 software was used to perform all statistical analysis (SPSS inc. 2005).

## Results

### Hematological and biochemical markers of Fe metabolism

Results from the measurement of hematological and biochemical parameters are presented in Table 1. According to the data obtained, all participants in this study showed a normal Fe metabolism with no biochemical and hematological evidence of Fe overload or deficiency. Significant differences between genders were found in Hb, total count of RBC, MCHC, Ft, and CP. In contrast, no association between age and hematological or biochemical markers of Fe metabolism was found in all individuals studied.

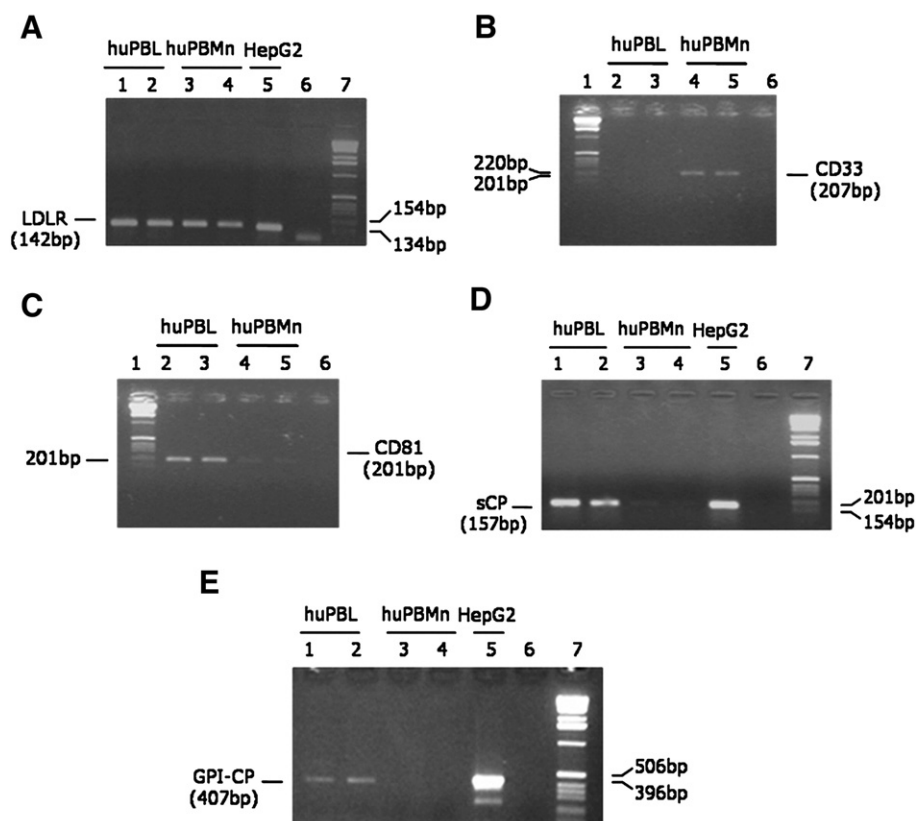


Fig. 1. RT-PCR results in human PBMC and HepG2 cells. Total RNA was extracted from isolated huPBL, huPBMn, and HepG2, followed by RT-PCR analysis. All RT-PCR products and 1 Kb ladder were separated in a 1.5% agarose gel at 80 V for 40 min. (A) LDLR RT-PCR: huPBL (lanes 1 and 2), huPBMn (lanes 3 and 4), HepG2 (lane 5), and blank (lane 6). (B) CD33 RT-PCR: huPBL (lanes 2 and 3), huPBMn (lanes 4 and 5), and blank (lane 6). (C) CD81 RT-PCR: huPBL (lanes 2 and 3), huPBMn (lanes 4 and 5), and blank (lane 6). (D) sCP RT-PCR: huPBL (lanes 1 and 2), huPBMn (lanes 3 and 4), HepG2 (lane 5), and blank (lane 6). (E) sCP RT-PCR: huPBL (lanes 1 and 2), huPBMn (lanes 3 and 4), HepG2 (lane 5), and blank (lane 6). In all cases, the amplification product was of the expected size.

*PBL transcriptional expression of secreted and membrane GPI-anchored ceruloplasmin*

RT-PCR was used to evaluate the transcriptional expression of CP in huPBL. For this purpose, cDNA samples of huPBL were prepared and tested by PCR using primers specific for LDLR, CD81, CD33, sCP, and GPI-CP. Human peripheral blood monocyte (huPBMn) cDNA samples were also prepared as positive controls for CD33 PCR assay. HepG2 cDNA sample was used as a positive control for specific amplification of sCP [31] and GPI-CP [36].

The integrity of all cDNA samples was analyzed in a PCR assay for LDLR with successful amplification of all samples (Fig. 1A). HuPBL and huPBMn samples were tested by PCR for their specific markers (CD81 and CD33, respectively),

confirming the successful isolation of both huPBL and huPBMn used for preparation of cDNA samples (Figs. 1B and C). HuPBL, huPBMn, and HepG2 samples were then tested for sCP and GPI-CP. As shown in Fig. 1, there was a successful amplification for both sCP (Fig. 1D) and GPI-CP (Fig. 1E) in huPBL and HepG2 samples. No amplification for GPI-CP transcript was detected in huPBMn samples while only a slight amplification was observed for sCP. This latter result is consistent with Mazumder *et al.*'s observation that sCP transcriptional expression is insignificant in resting monocytes [15], excluding a putative contribution of contaminating monocytes in huPBL samples.

The sequence of the amplified fragments was analyzed by DNA sequencing and compared to the nucleotide sequence for CP cDNA previously reported [31,37,38]. The

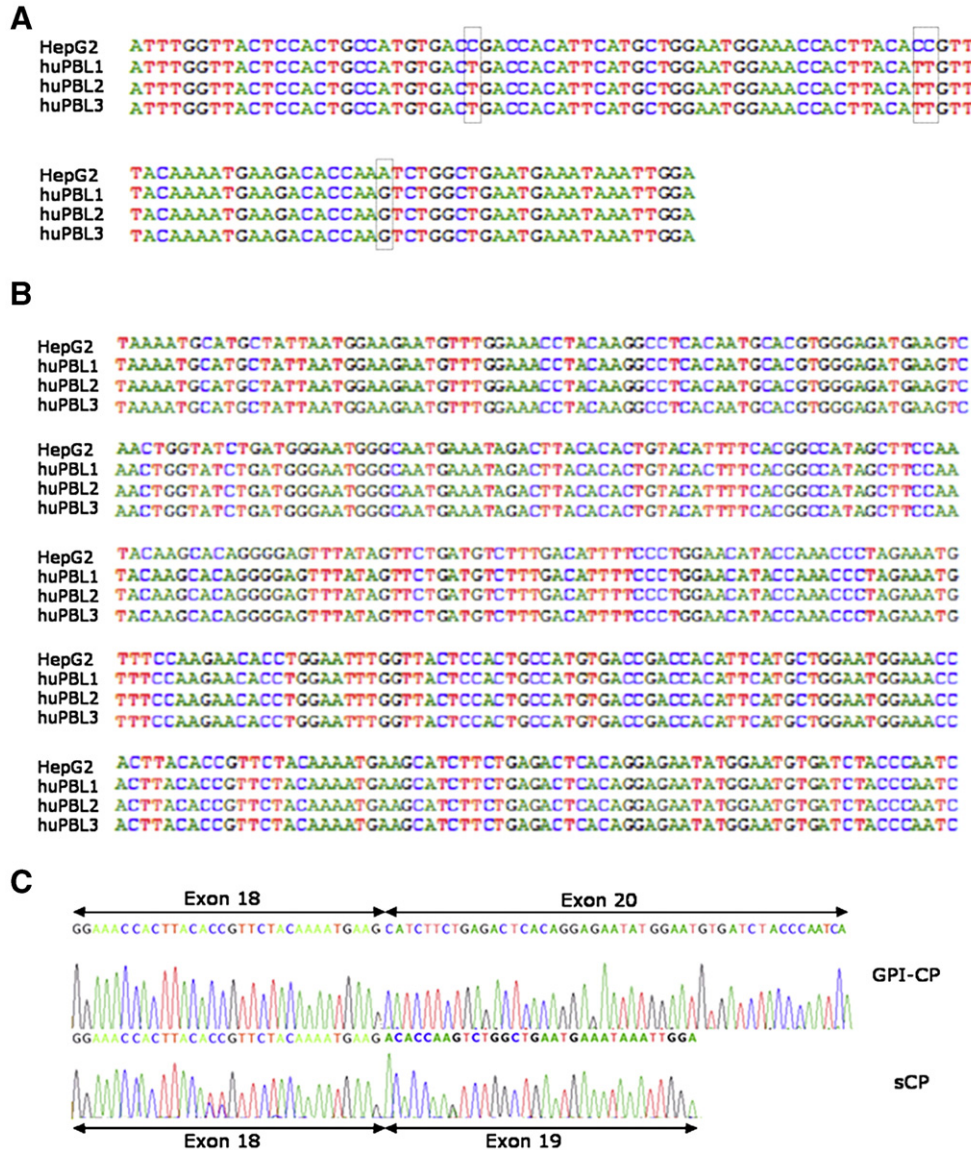


Fig. 2. Sequencing analysis of RT-PCR products for CP isoforms in huPBL and HepG2 cells. Total RNA was extracted from isolated huPBL and HepG2 followed by RT-PCR for GPI-CP and sCP. Amplified fragments were analyzed by DNA sequencing. (A) sCP sequence alignment for huPBL and HepG2 samples: polymorphic regions are shown in boxes. (B) GPI-CP alignment for huPBL and HepG2 samples. (C) Comparison between GPI-CP and sCP amplified sequences.

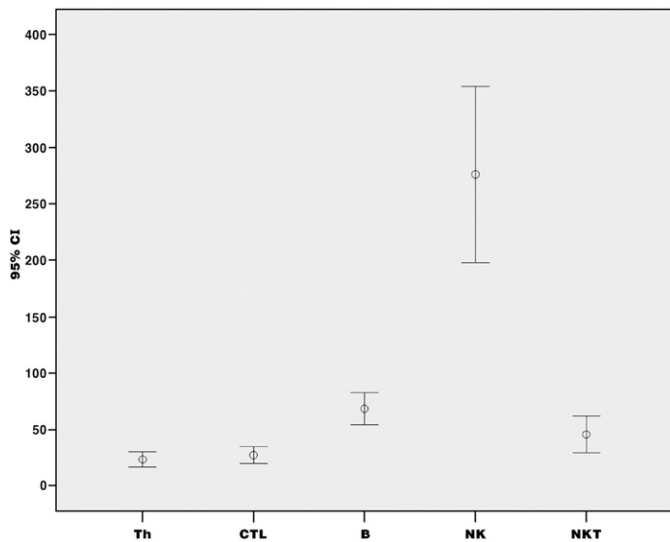


Fig. 3. Expression of CP in huPBL subsets: Th (CD3+CD4+),  $n=17$ ; CTL (CD3+CD8+),  $n=17$ ; B lymphocytes (CD3-CD19+),  $n=19$ ; NK cells (CD3-CD16+/CD56+),  $n=17$ ; NKT cells (CD3+CD16+/CD56+),  $n=18$ . Mean and respective 95% confidence intervals.

identification of sCP and GPI-CP fragments was confirmed, showing that huPBL express the transcripts for sCP and GPI-CP (Figs. 2A and B). Also, it showed a different 3' terminal sequence for CP transcripts (Fig. 2C) with possible origin at an alternative splicing mechanism as reported elsewhere [38,39].

#### *Ceruloplasmin expression by huPBL subpopulations has a high pattern of cell-type specificity*

Results obtained from flow cytometry analysis showed huPBL have differential CP expression according to the individual lymphocyte subset analyzed (Fig. 3). Importantly, despite that all the major lymphocyte subpopulations (Th, CTL, B, NK, and NKT lymphocytes) express CP, a highly cell-type-specific expression was observed on a NK lymphocyte subset (CD3-CD16+/CD56+ lymphocytes). Accordingly, a high correlation between CP expression in total huPBL and CP expressed in each lymphocyte subset was found, particularly with the NK cells subset (Pearson  $r=0.962$  and Spearman  $r_s=0.912$ ,  $P<0.001$ ). In fact, a good model fit was obtained for adjustment of NK cells expressing about 92% (adjusted  $R^2$ ) of variation of CP in total huPBL (Fig. 4). The hypothesis of Normally distributed residuals was not rejected (Kolmogorov–Smirnov test), and autocorrelation was not present.

Additionally, individual comparisons between CP expression in differential huPBL subpopulations showed a significant difference between the NK cells expressing CP and all the others subsets analyzed (Fig. 3). CP expressed in B lymphocytes and NKT cells was statistically different between them in comparison with all the other subsets. However, no significant difference was observed in the expression of CP between Th cells and CTL.

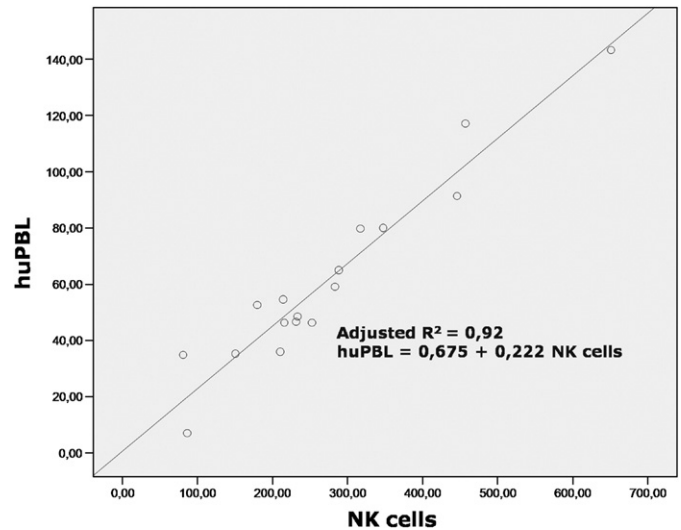


Fig. 4. Adjustment of NK cells to huPBL, using a linear regression model.

A search for putative correlations between hematological/biochemical parameters and CP concentration in serum and/or CP expression in huPBL showed that CP expressed at the huPBL surface is not correlated with serum Fe or serum Tf but is inversely correlated (Pearson  $r=-0.507$ ,  $P=0.02$ ) with serum Ft. Remarkably, no association was found between serum CP and its expression on the huPBL surface in all population analyzed.

#### *Human PBL express ceruloplasmin protein at its surface*

Nonpermeabilized huPBL cells were labeled for CP and imaged using confocal microscopy (Fig. 5). Observation of the immunolabeling in the different sections of resting huPBL (Figs. 5C to H) suggests that CP localizes essentially on the plasma membrane. In fact, the fluorescence intensity through the cell depicted in Fig. 6 is significantly higher on the cell periphery, giving further evidence for the presence of a CP membrane-bound isoform (Fig. 6).

## Discussion

In this study, we have shown that huPBL express two distinct CP transcripts, namely the secreted and the membrane GPI-anchored isoforms. Expression of CP in huPBL was confirmed at the protein level by flow cytometry analysis in all lymphocyte subpopulations studied. These observations are in agreement with previous detection of CP transcript in CD4+ cells, CD8+ cells and B lymphoblasts [32]. However, it is noteworthy to mention the significantly higher CP expression in NK cells compared to all other lymphocyte subsets analyzed. Moreover, flow cytometry and confocal microscopy analyses of immunolabeling on nonpermeabilized cells strongly indicate the presence of CP protein at the surface of huPBL. This cell surface staining of CP may indicate either expression of the membrane-bound GPI-CP form or binding of circulating CP. The statistical analysis showing no correlation between serum CP

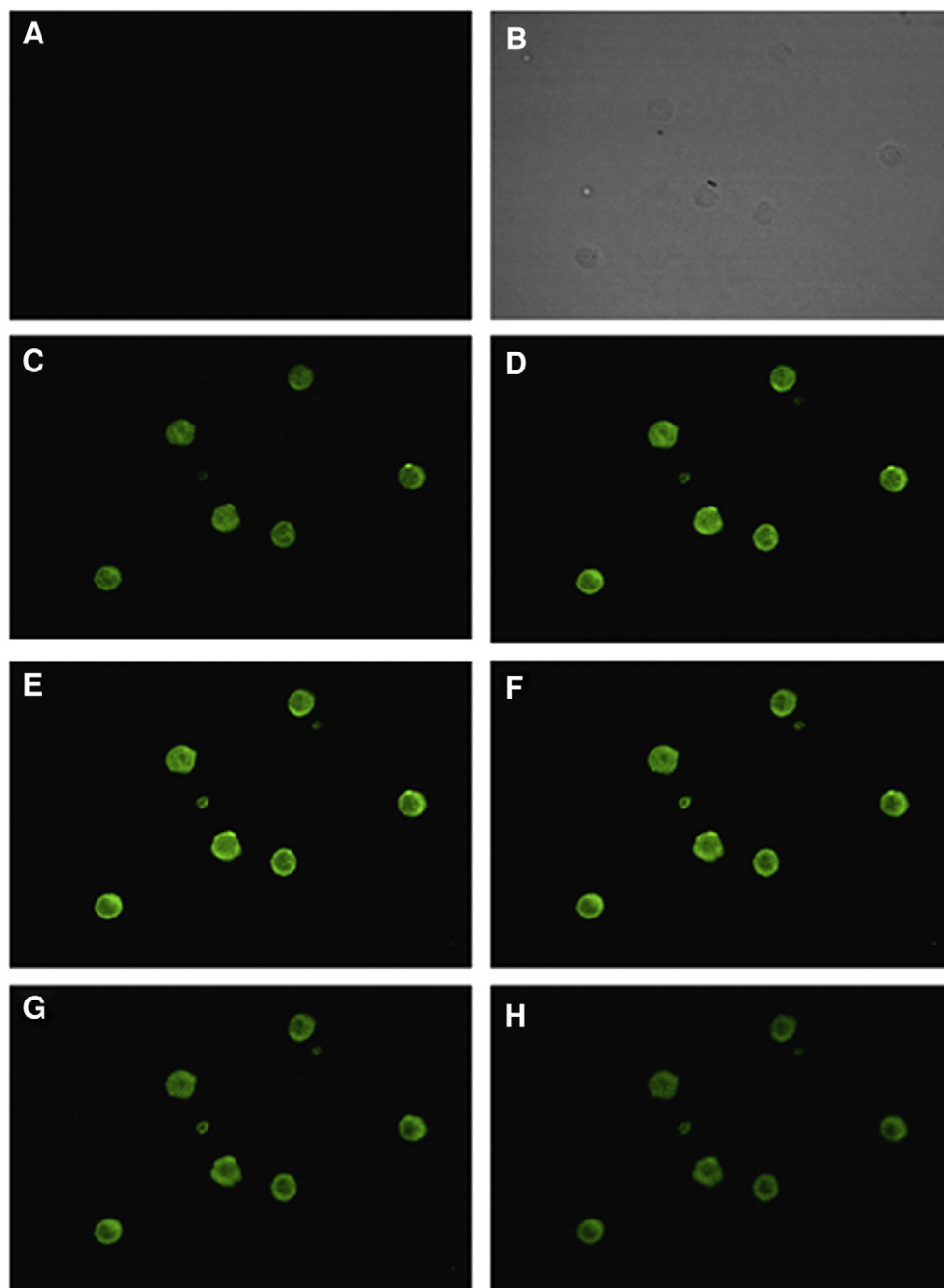


Fig. 5. Confocal imaging of CP immunolabelling in resting huPBL. Cells were fixed in 4% formaldehyde and stained with sheep anti-human CP-FITC conjugated (green): (A) unlabeled cells; (B–H) the same cells observed by phase contrast (B) or by fluorescence for CP in different confocal sections (B–H).

concentration and CP cell surface staining in these cells exclude, at least in part, the hypothesis of exogenous circulating CP binding and reinforce the hypothesis of GPI-CP protein expression in huPBL.

The role of CP ferroxidase activity on cellular Fe efflux and defense against oxidative stress has received considerable attention over the last years. Since its discovery, the source of circulating CP has been almost exclusively assigned to CP secreted by hepatocytes. However, CP transcripts produced by alternative splicing [40] were previously detected in different tissues. Human monocytic cells have also been shown to pro-

duce and secrete their own CP on activation [15]. Here, we have shown that huPBL express the transcripts for both CP molecular isoforms. During infection and inflammation characterized by active proliferation of circulating lymphocytes, CP concentration in serum increases, suggesting that the expression of the CP gene represents an essential part of host response to immunological stress [40]. Therefore, it will be of interest to study the relative contribution of PBL-derived CP to the increase of serum CP observed in specific physiological and pathological conditions. Since both sCP and GPI-CP transcripts were detected, one can speculate for either the direct secretion and/or cell



Fig. 6. Histogram reflecting the fluorescence intensity of CP through a line intersecting one section of a huPBL.

surface shedding of CP. It has been described that GPI proteins expressed at the cell surface of NK cells can be converted during activation to a soluble form in extracellular medium through a proteolytic cleavage involving a metalloprotease [41].

There is now compelling evidence that lymphocytes are involved in the regulation of Fe metabolism, namely through the synthesis or specific binding of several “Fe-related proteins” [42–45]. In this context, lymphocyte CP expression gives additional support to the original postulate from De Sousa *et al.* addressing a role for lymphocyte circulation in the regulation of Fe load [19] and establishes an additional link between immune system and Fe homeostasis.

In fact, lymphocytes have the capability to circulate, populate, and interact with cells and tissues. Interestingly, lymphocytes were also shown to synthesize the Fe(III) acceptor Tf [44]. A coordinated secretion of CP and Tf by huPBL may represent a protective function against the potential toxicity of Fe and its deposition in tissues. Indeed, CP may play an important role in the radical driven process and oxidative injury characteristic of Fe overload diseases such as HH [46]. Consistent with the role of CP as an endogenous antioxidant and scavenger of superoxide anion radicals, low numbers of local and/or circulating CP producing cells could predict a more severe presentation in HH patients and could also constitute an additional factor explaining clinical heterogeneity of the pathology. Several reports show that serum CP [47,48] and its ferroxidase activity [49] are decreased in HH patients. Moreover, defective numbers of huPBL were associated with a greater degree of Fe loading in HH [50] while Fe deposition and damage in liver from HH patients were shown to be associated with low numbers of CD8+ cells in the lobuli [51]. Interestingly, in healthy individuals liver-associated lymphocytes are characterized by a three-fold increase in the percentage of CD56+ cells (NK cells) and also an increase in the percentage of CD8+ cells compared to PBL [52]. Previous studies showed that the lowest numbers of NK cells were found in a group of HH patients with cirrhosis compared to asymptomatic patients [53] while a diminished NK activity in Fe overload conditions [54,55] has also been reported. Altogether, these observations underline a close link between Fe homeostasis and impairment of NK function. We have observed that NK cells are the subset of lymphocytes

presenting the highest expression of CP. In this context, it is therefore tempting to speculate that a decrease in lymphocyte population, especially in NK cells, could be involved in the pathophysiology of Fe overload, namely in hepatic tissues through a possible impairment of lymphocyte CP ferroxidase activity restricting proper Fe egress from cells.

An additional putative functional relationship between immune function and Fe metabolism through huPBL-derived CP could be related to the widely reported role of NK cells in infection and tumorigenesis. Previous studies have shown cellular Fe depletion as an effective host defense mechanism against pathogenic microorganisms and neoplasia [56], indicating a possible involvement for NK cell-derived CP in these processes. Increased levels of serum CP have been reported to occur in both human [57,58] and transgenic mice [59] hepatocellular carcinoma. The induction of CP production may represent a possible compensatory mechanism to control excessive cell proliferation by decreasing liver Fe due to its role on Fe egress from cells. Furthermore, through its protective function against oxidative stress, CP may also inhibit potential DNA damage and mutations which could potentiate tumor severity. The antitumor activity of CP [60] could be of special interest in HH considering the high incidence of cancer in these patients [61].

Alternatively, huPBL-derived CP physiological function may be directly related to its prooxidant activity. NK lymphocytes are known to have a key role on cell-mediated cytotoxicity. The potential mechanisms for cytotoxic damage to targets cells involve NK cells’ release of breakdown enzymes or other toxic substances able to cause profound cell damage. In this context, secretion or shedding of NK cell-derived CP may also contribute to cell-mediated host defense processes acting through its prooxidant properties in a manner analogous to the release of oxygen and nitrogen species. Indeed, CP was shown to present some bactericidal activity [62] and to suppress respiratory burst in phagocytic cells under Cu deficiency conditions [13]. On the other hand, the surface expression of the GPI-CP isoform on effector cells could function as a cytoprotective barrier in a harmful environment, namely through its antioxidant capacity.

In conclusion, several lines of evidence suggest a close functional relationship among the immune system, oxidative stress, and Fe metabolism through huPBL-derived CP. However, the precise role of CP expressed by lymphocytes on Fe metabolism and host defense mechanisms needs to be investigated. In particular, the physiological function assigned to the two specific CP isoforms (sCP or GPI-CP) expressed by huPBL needs to be clarified.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2007.10.032.

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