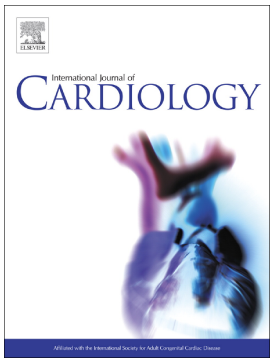


# Immature surfactant protein-B impairs the antioxidant capacity of HDL

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**Immature Surfactant Protein-B impairs the antioxidant capacity of HDL**

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All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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**Abbreviations**

ACE, angiotensin-converting enzyme; ACM, alveolar-capillary membrane; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoM, apolipoprotein M; AT1, angiotensin II subtype 1 receptor; BMI, body mass index; BNP, B-type natriuretic peptide; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; DCF, Dichlorofluorescein; DLCO, carbon monoxide lung diffusion; ESRD, end-stage renal disease; Hb, haemoglobin; HDL, high-density lipoprotein; HF, heart failure; HOI, HDL oxidant index; LCAT, lecithin: cholesterol acyl transferase; LC-MS, liquid chromatography-mass spectrometry; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro–B-type natriuretic peptide; NYHA, New York Heart Association; PLA2, phospholipase A2; PON-1, paraoxonase-1; proSP-B, immature surfactant protein B; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; SP-D, surfactant protein D; $VCO_2$, carbon dioxide production; VE, minute ventilation; VLDL, very low-density lipoprotein; $VO_2$, oxygen consumption.

**Abstract**

Circulating immature surfactant protein B (proSP-B) forms emerged as the most reliable lung-specific circulating marker for alveolar-capillary membrane (ACM) dysfunction and for the overall clinical status of heart failure (HF). Notably, in terms of HF hospitalization, immature SP-B overwhelms the prognostic role of other most frequently used clinical parameters such as those related to lung dysfunction. The strong prognostic value of circulating proSP-B in HF suggests more widespread and possible systemic effects. Thus, we assessed the plasma distribution of proSP-B evaluating whether it exists in a lipoprotein-bound form and its impact on lipoprotein structure and function.

ProSP-B forms were detectable in high-density lipoprotein (HDL) only. To assess the impact of proSP-B on HDL, HDL from healthy subjects were enriched with proSP-B produced by a stably transfected CHO cell line that specifically expresses and releases the human proSP-B. After enrichment, HDL size and lipoprotein electrophoretic mobility, and protein composition did not show apparent differences. HDL anti-oxidant capacity (HOI), assessed as their ability to inhibit air-induced LDL oxidation, was impaired after proSP-B enrichment. HOI was also higher in HF patients with respect to age-matched control healthy subjects ($p=0.013$).

Circulating proSP-B, besides its potential role as a specific marker for ACM dysfunction in HF patients with diagnostic and prognostic value, binds to human HDL impairing their antioxidant capacity. These findings shed light on proSP-B as a molecule that contributes to the reduction of the defense against oxidative stress, a key mediator in the pathogenesis of HF.

## 1. Introduction

Surfactant protein B (SP-B) is vital for normal lung function, and its complete deficiency leads to lethal, neonatal respiratory distress syndrome, which is characterized by a virtual absence of lung compliance, highly disorganized lamellar bodies, and greatly diminished levels of surfactant protein C (SP-C) mature peptide [1].

Our recent researches strengthen the role of the circulating immature SP-B forms (proSP-B) as the most reliable lung-specific circulating marker for alveolar-capillary membrane dysfunction (carbon monoxide lung diffusion (DLCO)) and for overall clinical status (New York Heart Association (NYHA) class, peak oxygen consumption (VO2), minute ventilation (VE)/carbon dioxide production (VCO2) slope, etc.) of HF [1, 2]. Notably, in terms of HF hospitalization, immature SP-B overwhelms the prognostic role of other most frequently used parameters related to lung dysfunction such as DLCO, VE/VCO2 slope and spirometric data [1]. With respect to other proteins proposed as possible markers of lung damage [3], immature circulating SP-B has some peculiarities that render it a potential specific marker for alveolar-capillary membrane dysfunction, such as: its essential role in the assembly of pulmonary surfactant; its predominant pulmonary synthesis (https://www.proteinatlas.org/ENSG00000168878-SFTPB/tissue), which differs from other surfactant proteins (i.e. surfactant protein A (SP-A) and surfactant protein D (SP-D)); its multistep pulmonary-cell-specific proteolytic maturation, which yields many immature intermediates with different molecular masses (from ~40 to ~20 kDa); and, its storage with surfactant phospholipids in lamellar bodies, the contents of which are released into the bloodstream only in case of alveolar–capillary barrier damage.

SP-B biosynthesis is a complex process involving both post-translational and proteolytic events. Prepro-SP-B is modified by glycosylation and signal peptide cleavage resulting in the proSP-B within the endoplasmic reticulum. Extensive studies demonstrated that the initial proteolytic cleavage of the N-terminal propeptide occurs in the medial Golgi with a subsequent C-terminal cleavage in the trans-Golgi, whereas a final N-terminal cleavage event occurs in a post-Golgi

compartment, possibly in the multivesicular body, resulting in the mature form of SP-B in the lamellar body [1].

SP-B has a strong hydrophobic character [4], it is water-insoluble, co-isolates with lipids during the extraction of surfactant with organic solvents, and, consisting of amphipathic $\alpha$-helices connected by highly apolar loops, preferably interacts with anionic phospholipids [4]. *In vitro*, addition of SP-B to liposomes, composed of synthetic phospholipids, leads to membrane binding, destabilization, and fusion, ultimately resulting in dramatic rearrangement of the membrane structure; two properties, fusion and destabilization, that are likely important for the transition of surfactant phospholipids from the intracellular stores to the extracellular surfactant film [5].

The strong prognostic value of circulating proSP-B in HF suggests more widespread and possible systemic effects. Thus, considering its lipophilic nature and high affinity for phospholipids, we assessed the plasma distribution of proSP-B, evaluating whether it exists in a lipoprotein-bound form and its impact on lipoprotein structure and function.

## 2. Methods

### 2.1. Patients and control subjects characteristics.

A subset of healthy subjects (controls) and HF patients was selected, according to their age, sex and clinical characteristics, from a population set described previously [2]. The study was approved by the Ethical Committee European Institute of Oncology and Monzino Cardiologic Center, (registration number R454/16-CCM470) [2] and complied with the Declaration of Helsinki.

**Supplementary Table 1** shows the clinical characteristics of the subjects. Lipoproteins were isolated by sequential ultracentrifugation as previously described [6].

**2.2. Generation of stably transfected proSP-B–expressing CHO cells.** The pcDNA5/FRT:SP-B/Ile, constructed as described [7], or an empty pcDNA5/FRT vector were transfected into Flp-In-CHO cells (R758-07, Invitrogen, Carlsbad, CA, USA) together with a pOG44 expression vector

(V6005-20, Invitrogen, Carlsbad, CA, USA) and the proSP-B–expressing clones were selected according to the Flip-In System protocol (K6010-01, Invitrogen, Carlsbad, CA, USA). All experiments were performed using cells within 20 passages from transfection and selection.

**2.3. Preparation of proSP-B-enriched solution.** Conditioned media derived from proSP-B–expressing CHO (~$1*10^8$ cells) were processed as described [8]. Briefly, after collection, media were centrifuged for 5 min at 1000 x *g* at 4°C to remove cell debris, and the clear supernatant was dialyzed at 4°C using a 3500 Da molecular weight cut-off dialysis tube (Spectrum Laboratories, Rancho Dominguez, CA, USA) against 1 mmol/L $NH_4HCO_3$ at 4 °C for 4 h, 0.75 mmol/L $NH_4HCO_3$ for 4 h, 0.5 mmol/L $NH_4HCO_3$ for 16 h, 0.25 mmol/L $NH_4HCO_3$ for 4 h, and in last step against distilled water for 4 h. The samples were then lyophilized and stored at -80°C. These concentrated media were dissolved in water and used to enrich high-density lipoprotein (HDL) from control subjects with proSP-B. After preliminary experiments, HDL incubation with proSP-B enriched media was performed following a protein ratio 1:10 to achieve a proSP-B immunoreactivity of enriched HDL similar to that observed with HDL isolated from HF patients.

**2.4. Secretome analysis.** For the analysis of cell secretome by label-free mass spectrometry, the conditioned media were collected from cells and processed as described [8]. Before tryptic digestion, the samples were dissolved in 25 mmol/L NH4HCO3 containing 0.1% w/v RapiGest SF, reduced with 5 mmol/L dithiothreitol, dissolved in 100 mmol/L NH4HCO3, at 60 °C for 15 min, and then carbamidomethylated with 10 mmol/L iodoacetamide for 30 min at room temperature. Digestion performed overnight at 37 °C using sequencing grade trypsin (Promega, Milan, Italy) was stopped by the addition of 2% v/v TFA. Quantitative label-free LC-$MS^E$ was performed as previously described [9, 10]. The proteins were identified by searching a *Cricetulus Griseus* UniProt database (release 2017.9; 35169 unreviewed entries), with the addition of the sequences of human SP-B and alcohol dehydrogenase (ADH) from *Saccharomyces Cerevisiae*, as internal

standard for molar estimation. Quantitation was performed using Progenesis QIP for proteomics (v3.0, NonLinear dynamics, Newcastle upon Tyne, UK).

**2.5. Immunoblotting of proSP-B.** Lipoprotein samples, cells and culture media were assayed for proSP-B expression and secretion by immunoblotting analysis as previously described [2]. Briefly, proteins (50 µg) were separated by one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels in Tris-Tricine buffer in non-reducing conditions and then transferred to nitrocellulose at 60 V for 2 h. Immunoblotting was performed as follows: blocking in 5% weight/volume nonfat milk in Tris-buffered saline (100 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) containing 0.1% Tween-20 for 1 h at room temperature; overnight incubation at 4°C with primary antibody against SPB (rabbit anti-human SP-B H300, sc-13978, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:400 in 5% weight/volume nonfat milk in Tris-buffered saline with 0.1% Tween-20; and incubation with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (170-5046, Bio-Rad, Hercules, CA, USA) at 1:5000 for 1 h. Bands were visualized by enhanced chemiluminescence using the enhanced chemiluminescence kit (RPN2109, GE Healthcare, Buckinghamshire, UK).

**2.6. HDL Oxidant Index (HOI).** HOI was measured using a Dichlorofluorescein (DCF)-based fluorescent assay, as described [11], in lipoprotein isolated from plasma of healthy subjects using a precipitation-based method [12].

**2.7. Characterization of proSP-B enriched HDL**. HDL characterization was performed by SDS-PAGE (4-20% polyacrylamide SDS gels) and, by gradient gel electrophoresis (4-20% non-denaturing polyacrylamide gels for 3.5 h at 200 V, 4°C). The apolipoprotein composition of HDL was determined by densitometric analysis of Coomassie-stained SDS 4-20% polyacrylamide

gradient gels on which 5 µg aliquots of HDL samples were analyzed. Individual apolipoprotein values were expressed as a percentage of the total value of all proteins in the lane.

The electrophoretic mobility of the HDL was determined on agarose gels (1%) in Tris 0.5 mol/L (pH 8.6). Sample loading was 5 µg HDL per lane, and electrophoresis was carried out for 90 min at 100 V. Visualization was done by staining with Sudan Black B (0.1% w/v) in 70% ethanol.

**2.8. Statistical analysis**. Data are expressed as means ± SD. HOI data were analyzed using GraphPad Prism (version 5) and were statistically compared using paired, two-tailed Student's t-test (8 independent experiments to obtain power>80% for HDL enriched with proSP-B), or unpaired two-tailed Student's t-test for HDL from patients and controls, after normality assessment by Kolmogorov–Smirnov test. P values of <0.05 were considered significant.

## 3. Results

To analyse the distribution of proSP-B in the lipoprotein classes, we isolated VLDL, LDL, and HDL by ultracentrifugation from the plasma of HF patients (n=5) with the highest circulating levels of immature proSP-B (>20 AU) selected from the sample set described previously [2]. All individuals were males (age 59±16 years) with the following clinical characteristics: NYHA class III, DLCO <80%, B-type natriuretic peptide (BNP) >160 pg/ml, peak VO2<15 ml/Kg [2]. Lipoproteins were isolated also from five age- (54±1 years) and sex-matched control subjects to compare proSP-B distribution. Herein, we demonstrated that proSP-B forms (from ~40 to ~20 kDa) were detectable in HDL only, and not in VLDL, LDL (**Fig. 1A**), or in the lipoprotein depleted plasma, with higher immunoreactivity in HDL from patients than those from controls. The bands at ~20 kDa and ~25 kDa are not consistently present in controls and patients, likely reflecting individual variability (**Fig. 1A and 1C**).

We then assessed the impact of proSP-B on HDL structure and function, in terms of antioxidant property. Because the immature proSP-B forms are difficult to be purified from the lung surfactant,

we enriched HDL from control subjects (n=8) by incubating them with desalted and concentrated supernatant derived from a stably transfected CHO cell line that specifically expresses and releases the human proSP-B (**Fig. 1B**). Mass spectrometry analysis confirmed that secretome from CHO overexpressing proSP-B cells does contain this protein, whilst that from control CHO does not. Specifically, the amount of released proSP-B was 150±28 ng (from three independent biological replicates) in a total of 100 μg of secreted proteins, other proteins (26 proteins) from *Cricetulus Griseus*, identified and quantified by mass spectrometry, were not different between the two transfected cells types (**Supplementary Table 2**). After incubation with the cell culture media derived from the CHO cells transfected with an empty vector (control cells), or CHO overexpressing proSP-B, HDL were re-isolated by ultracentrifugation and were found to be enriched with proSP-B, even after 2 hours of incubation (**Fig. 1C**).

HDL size, assessed by non-denaturing gradient gel electrophoresis (**Fig. 1D**), and by electrophoretic mobility on agarose gel (**Fig. 1E**), did not show apparent differences even after prolonged (24 h) proSP-B enrichment. No significant differences were observed neither in HDL protein composition; specifically, in the apolipoprotein A-I (apoA-I) content of control HDL and proSP-B-enriched HDL (69.5±5.1% vs. 66.8±4.7%, respectively), or in the apolipoprotein A-II (apoA-II) content (5±0.6% vs. 5.7±0.7%, respectively). We then evaluated HDL anti-oxidant capacity by assessing their ability to inhibit air-induced LDL oxidation, which was measured by DCF fluorescence as described [11]. In the assay, the change in fluorescence intensity is the result of the oxidation of DCFH induced by free radicals generated in the oxidation of human LDL in the absence or presence of HDL. The DCF fluorescence data were converted into an HOI that equalled the ratio of fluorescence in the presence of HDL divided by the fluorescence in the absence of HDL. We found that HDL derived from control subjects, enriched with proSP-B for 2 hours, showed a significant reduction in their ability to inhibit oxidation of LDL by air with respect to HDL incubated with the control CHO cells media (**Fig. 1F** and **1G**). This finding suggests that proSP-B associated to HDL acts, at least in part, as a player in HDL dysfunctionality due to impaired anti-oxidant capacity. HOI was also higher in

HF patients with respect to age-matched control healthy subjects (**Supplementary Table 1** and **Fig. 2**).

## 4. Discussion

We have previously demonstrated that plasma levels of immature proSP-B were higher in HF patients than in controls [2]. We showed herein that circulating proSP-B, besides its potential role as a specific marker for alveolar capillary membrane dysfunction in HF patients with diagnostic and prognostic value, binds to human HDL particles impairing their anti-oxidant capacity. Our findings extend recent observations which showed that higher level of SP-B (not specifying if immature or mature proteoform) in a lipid-enriched fraction (likely reflecting HDL composition) could be a better predictor of HF outcome [13]. Indeed, we demonstrated that circulating proSP-B is not present in a free form, it exclusively binds to isolated HDL, and it impairs HDL anti-oxidant properties. These hypothesis-generating findings shed light on proSP-B proteoform as a molecule that contributes to the reduction of the defense against oxidative stress, a key mediator in the pathogenesis of HF [14]. Indeed, an involvement of oxidative stress in the left ventricular dysfunction and cardiac remodelling, likely leading to impaired excitation-contraction coupling, extracellular matrix remodelling, hypertrophy of cardiomyocytes and myocardial cell apoptosis, has been well established [14]. Further, in these conditions, HDL lose their protective properties becoming functionally impaired and even promoting pro-oxidative processes [15].

The failure of the HDL-cholesterol-raising therapies to improve outcome in patients with coronary artery disease, based on the observed inverse correlations between the HDL-cholesterol levels and the risk for development of cardiovascular diseases, paved the way to the hypothesis that not simply quantity but rather functions and composition of HDL may be more relevant for the protection in cardiovascular diseases [15].

Furthermore, Patel *et al* demonstrated that patients with stable, chronic HF have impaired HDL anti-oxidative capacity as well as reduced cholesterol efflux capacity [16]. A recent study extended

this initial observation and, showed that an impaired HDL anti-oxidative capacity is associated with higher mortality in HF patients independently of the traditional cardiovascular risk factors and irrespectively of the underlying etiology [17].

On this topic, recent advancement in proteomics technology has dramatically increased our understanding of proteins carried by HDL. In addition to proteins with well-established functions in lipid transport, iron transport proteins, members of the complement pathway, as well as proteins involved in immune function and acute phase response were repeatedly identified on HDL particles. In a pathological state, various protein and lipid components of the HDL can undergo alterations, which drive a shift towards a dysfunctional state of the lipoprotein becoming, pro-oxidant, pro-inflammatory, and lastly pro-atherogenic [6, 18, 19].

During the acute phase response, for example, circulating serum amyloid A displaces Apo A-I and incorporates into the lipoprotein membrane, becoming one of the main protein components of HDL, and impairing HDL's anti-oxidative functionality. *Vice versa*, clusterin decreased in HDL isolated from patients with coronary artery diseases, leading to activation of pro-apoptotic signalling pathways [20].

The concept that additional components of HDL might be responsible for impaired atheroprotective functions emerged from the observation that association of poor long-term prognosis in patients with HF and low paraoxonase-1 (PON-1) activity, which was considered the main responsible for the anti-oxidant effects of HDL, was significant only in unadjusted Cox regression analysis, but not after adjustment for other confounders in multivariable model including HOI, in addition to age, sex, NYHA classification, N-terminal pro-B-type natriuretic peptide (NT-proBNP), left ventricular ejection fraction, estimated glomerular filtration rate, body mass index, HDL, C-reactive protein, paraoxonase activity, diabetes mellitus, and atrial fibrillation [17]. Further, HOI resulted to be a more robust and independent risk predictor, even after adjustment for NT-proBNP and other potential confounders, and it was able to improve risk stratification obtained with NT-proBNP or the multiple variables described above [17]. Thus suggesting that PON-1 is not the only component

of HDL that sustains the anti-oxidant activity. Among known HDL factors potentially able to protect LDL such as apolipoprotein A-I, apolipoprotein J, platelet-activating factor acetylhydrolase, lecithin-cholesterol acyltransferase, and glutathione selenoperoxidase, we suggest that proSP-B can contribute to the dysfunctional state of HDL.

Some study limitations need to be acknowledged. First, we limited our investigations on the detrimental effect of proSP-B on HDL anti-oxidant capacity but we cannot exclude that other HDL functions, such as anti-inflammatory properties, can be affected by proSP-B. Indeed, we evaluated HDL anti-oxidant capacity because this is considered a good predictor of cardiovascular health outcomes [16]. To this end, we took advantage of the method developed by Navab *et al.* [21], which allows a widespread testing of the hypothesis that HDL is dysfunctional and provides identical results as from an established cell-based assay [21].

Second, we do not know how proSP-B can impair the anti-oxidative properties of HDL and there is a lack of knowledge regarding the physical binding of proSP-B to HDL. The lack of the effect of proSP-B on HDL size, although assessed only by gel electrophoresis, might be due to the low molecular weight of the protein or to a possible saturation kinetic, or both. Concerning the mechanism(s) through which proSP-B impairs HDL anti-oxidant capacity, we can only speculate that the interaction of proSP-B with others anti-oxidant proteins might alter the critical environment for their enzymatic activity to take place. To this regards, there are known players that can be responsible for the anti-oxidative properties of HDL, such as PON-1, apoA-I, apolipoprotein M (apoM), and phospholipase A2 (PLA2) (reviewed in [22]). Acting together on HDL, these factors probably create a system with relevant anti-oxidative properties. For example, apoA-I is essential for both the structure of HDL and the maintenance of the lipid environment in which enzymes such as PON-1 and lecithin: cholesterol acyl transferase (LCAT) can operate. Indeed, the interaction of lipids with apoA-I in HDL provides a lipoprotein particle capable of acquiring potentially toxic lipids and holding them in an environment where they may be safely hydrolysed and from which they may be released to the liver for elimination. On the contrary, there is evidence from both

animal models and human studies to suggest that apoA-II might actually suppress PON-1 binding to HDL [23]. To increase the complexity of this scenario, PON-1, PLA2, and LCAT are present at higher concentrations in small, dense, protein-rich HDL [24], and HDL particles are therefore heterogeneous in their anti-oxidative capacity. For this reasons, instead of analysing a specific factor (i.e. PON-1) we adopted the assay developed by Navab *et al* [21], which allows widespread testing of the hypothesis that HDL is dysfunctional and provides identical results as from an established cell-based assay [25].

Third, although circulating proSP-B represents a factor able to impair HDL protective functions through its selective binding to this lipoprotein, more data are needed to explain the relevant pathophysiology, with at least two possible pathways that might even superimpose: a) a primary lung damage could possibly induce, through the proSP-B release and its linkage to HDL, a further systemic damage, leading eventually to HF. Notably, a primary lung dysfunction, such as chronic obstructive pulmonary disease (COPD), worsen a concomitant HF [26]; b) HF is able by itself to induce damage to alveolar-capillary barrier with consequent proSP-B release [1], thus potentially implying a loop of progressive HF worsening due to impaired anti-oxidant function of HDL particles.

Finally, proteomic studies found SP-B, not specifically proSP-B, to be associated to HDL fraction in patients with coronary artery disease and in end-stage renal disease (ESRD) patients [20, 27]; in the latter, the plasma SP-B was similar to the controls. Thus, it becomes urgently needed to develop a quantitative, high-throughput mass spectrometry-based assay, which is so far not available, to validate proSP-B proteoform as a disease marker in the cardiovascular field.

## 5. Conclusions

In conclusion, circulating proSP-B, in addition to being the most reliable lung-specific circulating marker for alveolar capillary membrane dysfunction and for the overall clinical status of HF, is able to impair HDL protective functions through its selective binding to this lipoprotein.

Consequently, proSP-B measurement might help to develop strategies to identify patients at high risk for cardiovascular events and, may help to tailor treatment to the individual patient. Indeed, identification of novel predictive markers of mortality and cardiovascular events might help to improve the knowledge of the underlying pathophysiologic mechanisms of HF.

**Source of findings**

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**Disclosures**

None

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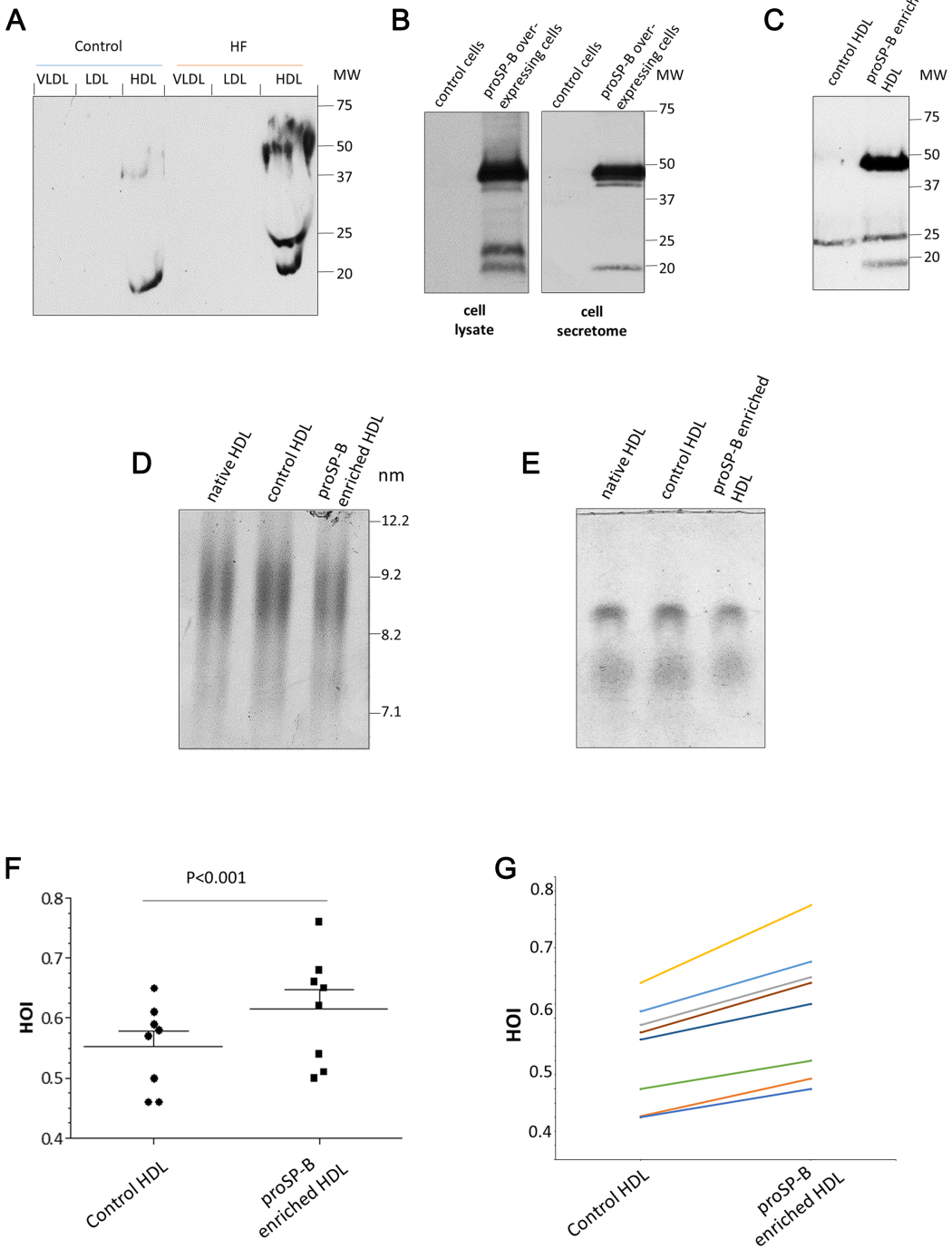
**Legends for Figures**

**Fig. 1: Distribution of proSP-B in human lipoproteins and effects of the proSP-B enrichment on HDL size and anti-oxidative capacity. A:** Distribution of proSP-B (from ~40 to ~20 kDa) in human lipoproteins. VLDL (d=1.006 g/ml), LDL (d=1.020-1.63 g/ml) and HDL (d=1.063–1.21 g/ml) were isolated by sequential ultracentrifugation from the plasma of control subjects or HF patients and subjected to immunoblotting analysis. The image is representative of three independent experiments. **B:** proSP-B immunoblotting analysis of the cell lysate and conditioned media (cell secretome) derived from CHO cells stably transfected with pOG44 and pcDNA™5/FRT empty vector (control, lane 1 for each image) or with the pcDNA5/FRT:SP-B/Ile construct (lane 2 for each image). The images are representative of five independent experiments. **C:** HDL isolated from control subjects (n=8) were incubated for 2 hours at room temperature under gentle agitation with conditioned media from control CHO (control HDL, lane 1) or CHO overexpressing proSP-B (proSP-B enriched HDL, lane 2). HDL were re-isolated by ultracentrifugation and analyzed by immunoblotting against SP-B. The image is representative of three independent experiments. **D, E:** Non-denaturing gel electrophoresis and agarose gel of native HDL (lane 1), control HDL (lane 2) and proSP-B enriched HDL (lane 3), respectively. The image is representative of three independent experiments. Molecular mass markers used in panel D were ferritin (440 kDa, ~12.2 nm), catalase (232 kDa, ~9.2 nm), lactate dehydrogenase (140 kDa, ~8.1 nm), and albumin (67 kDa, ~7.1 nm). **F:** HDL oxidant index (HOI) for control HDL and proSP-B enriched HDL. HOI was calculated as the ratio of DCF fluorescence in the presence of HDL divided by the fluorescence in the absence of HDL. Data were obtained with HDL from eight control subjects analysed in quadruplicate. The **G** panel highlights the behaviour of individual HDL before and after proSP-B enrichment.

**Fig. 2: HDL oxidant index (HOI) in controls and HF patients.** Data are expressed as mean±SD.

**Highlights**

- proSP-B is a potential diagnostic and prognostic circulating HF marker
- It binds to human HDL particles impairing their antioxidant capacity
- proSP-B might contribute to the reduction of defense against oxidative stress in HF


A
Control
HF
VLDL
LDL
HDL
VLDL
LDL
HDL
MW
75
50
37
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B
control cells
proSP-B over-expressing cells
control cells
proSP-B over-expressing cells
MW
75
50
37
25
20
cell lysate
cell secretome
C
control HDL
proSP-B enriched HDL
MW
75
50
37
25
20
D
native HDL
control HDL
proSP-B enriched HDL
nm
12.2
9.2
8.2
7.1
E
native HDL
control HDL
proSP-B enriched HDL
F
P<0.001
HOI
0.8
0.7
0.6
0.5
0.4
Control HDL
proSP-B enriched HDL
G
HOI
0.8
0.7
0.6
0.5
0.4
Control HDL
proSP-B enriched HDL

Figure 1

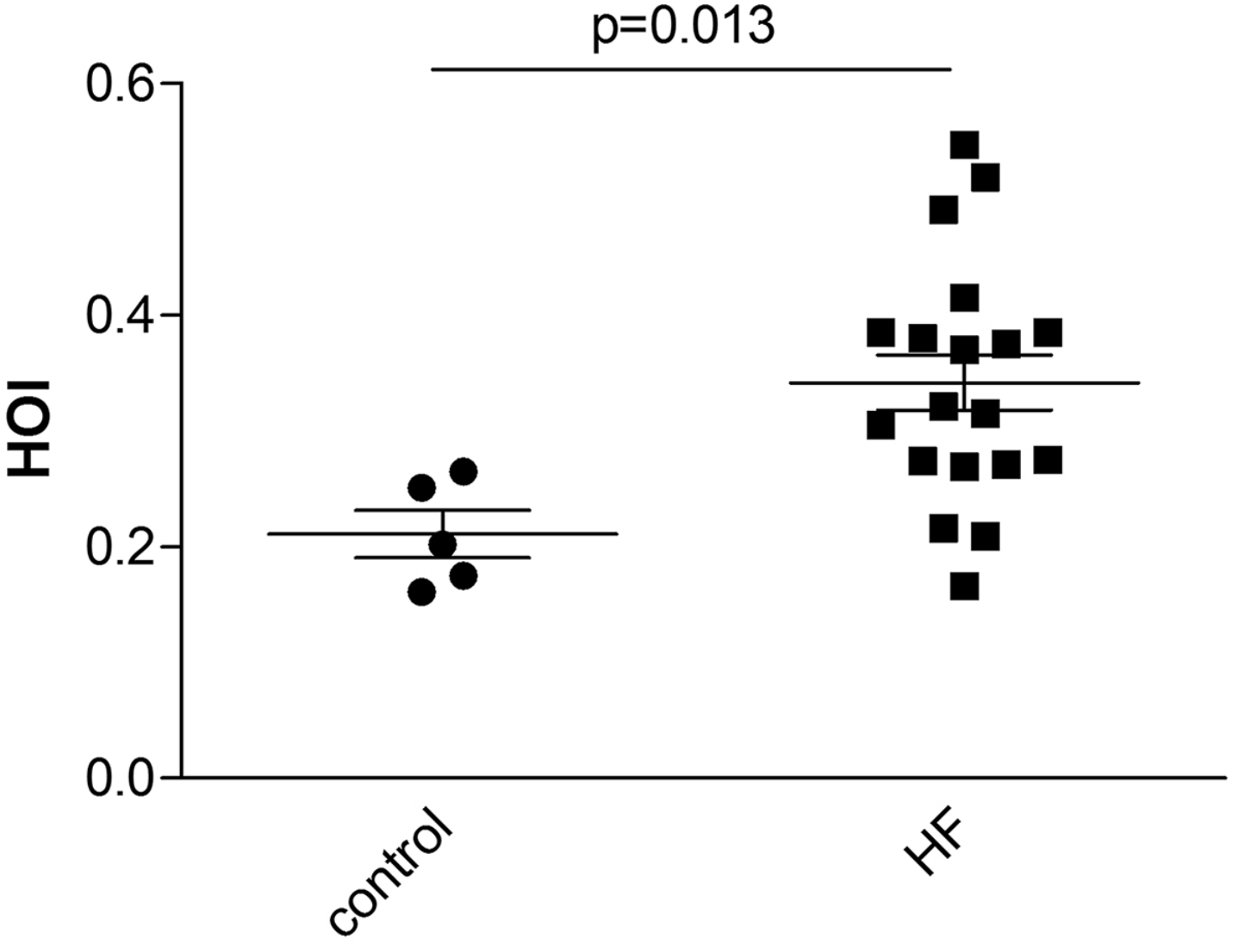


Figure 2