

# Increased levels of proteins of the acute inflammatory phase in the peritoneal fluid of women with advanced stages of endometriosis

Zwiększone stężenie białek ostrej fazy w płynie otrzewnowym kobiet z zaawansowaną endometriozą

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## Abstract

**Objectives:** Most investigators agree that endometriosis is associated with a state of subclinical, non-infectious peritoneal inflammation. The objective of the study was to assess concentrations of two markers of the acute inflammatory phase proteins, haptoglobin and ceruloplasmin, in peritoneal fluid of endometriotic women.

**Material and methods:** 229 women who underwent diagnostic or therapeutic laparoscopy were included in the study. Minimal, mild, moderate and severe endometriosis according to ASRM was confirmed in 119 women (study groups), whereas 110 patients suffered from simple serous or dermoid ovarian cysts (reference groups). Haptoglobin and ceruloplasmin concentrations in the peritoneal fluid samples aspirated during laparoscopy were measured using commercially available radial immunodiffusion kits.

**Results:** The concentration of haptoglobin in the peritoneal fluid of women with endometriosis was significantly higher as compared to patients with serous and dermoid ovarian cysts. Significantly higher haptoglobin level was observed in patients with severe and moderate endometriosis as compared to women from both reference groups. No significant difference in the peritoneal fluid ceruloplasmin levels was found between patients with endometriosis and women from reference groups. However, it was noted that ceruloplasmin levels are higher in the subgroup of patients with severe endometriosis as compared to both reference groups and women with mild disease.

**Conclusions:** Our results support the hypothesis that endometriosis is associated with subclinical inflammation within the peritoneal cavity. It may be speculated that pro-inflammatory stimuli strong enough to cause an increase in acute inflammatory phase proteins peritoneal fluid concentrations are observed only in the advanced stages of the disease.

Key words: **acute inflammatory phase proteins / endometriosis / haptoglobin / ceruloplasmin /**

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## Streszczenie

**Cel pracy:** Etiopatogeneza endometriozy nie jest do końca wyjaśniona, a większość badaczy zgadza się, że w płynie otrzewnowym kobiet chorych na tę chorobę stwierdza się subkliniczny proces zapalny pochodzenia nie-infekcyjnego. Celem pracy była ocena stężenia dwóch markerów, białek ostrej fazy zapalnej: haptoglobiny i ceruloplazminy w płynie otrzewnowym kobiet z endometriozą.

**Materiał i metody:** Do badań włączono 229 kobiet, które przebyły diagnostyczną lub terapeutyczną laparoskopię. Endometriozę w stopniu minimalnym, łagodnym, umiarkowanym wg ASRM potwierdzono histologicznie u 119 kobiet (grupa badana). Pozostałe 110 pacjentek leczono z powodu prostych lub dermoidalnych torbieli (grupa referencyjna). Stężenia haptoglobiny i ceruloplazminy w próbkach płynu otrzewnowego oceniano przy użyciu komercyjnych zestawów do immunodyfuzji radialnej.

**Wyniki:** Stężenie haptoglobiny w płynie otrzewnowym pacjentek z endometriozą było znacznie wyższe w porównaniu do pacjentek z grupy referencyjnej. Większe stężenie haptoglobiny dotyczyło podgrupy pacjentek z umiarkowaną i ciężką postacią choroby. Nie stwierdzono różnic w stężeniu ceruloplazminy w płynie otrzewnowym pacjentek z endometriozą w porównaniu do pacjentek z grupy referencyjnej. Jednakże wykazano, że stężenie ceruloplazminy jest większe w podgrupie pacjentek z ciężką postacią choroby w porównaniu do pacjentek z grupy referencyjnej i podgrupy pacjentek z łagodną postacią choroby.

**Wnioski:** Wyniki naszych badań potwierdzają hipotezę o istnieniu subklinicznego stanu zapalnego w jamie otrzewnowej pacjentek z endometriozą. Wydaje się, że działanie silnych bodźców prozapalnych powodujących zwiększenie stężenia białek ostrej fazy w płynie otrzewnowym dotyczy jedynie pacjentek z zaawansowanymi postaciami choroby.

Słowa kluczowe: białka ostrej fazy / endometrioza / haptoglobina / ceruloplazmina /

## Introduction

Despite the high prevalence and extensive investigations, the pathophysiology of endometriosis still remains enigmatic. However, almost all investigators dealing with the topic agree that endometriosis is associated with a state of subclinical, non-infectious peritoneal inflammation with peritoneal fluid (PF) immunological and angiogenic factors playing critical role in the disease pathogenesis. PF of women with endometriosis contains an increased number of activated macrophages, which exert phagocytic activity, produce and secrete numerous cytokines and growth factors. The specific function of these compounds is quite complex, and includes e.g. proliferation and differentiation of immunocompetent cells; hormones, enzymes, and acute inflammatory phase proteins release stimulation; immunoglobulins secretion regulation, chemotaxis and cytotoxicity mechanisms control [1]. Also, the concentrations of cytokines and growth factors are different in women with endometriosis as compared to healthy individuals. Enhanced PF cytokines concentrations in women with endometriosis reflect their increased production and secretion by peritoneal resident macrophages, lymphocytes, endometriotic implants and peritoneal cells [2].

Human ceruloplasmin, is a protein exhibiting copper-dependent oxidase activity and was first isolated from serum  $\alpha_2$ -globulins in 1948 [3]. The 134-kDa protein is comprised of a single polypeptide chain of 1046 amino-acid residues that tightly binds up to 6 copper atoms. Under physiological conditions, it binds up to 95% of circulating copper [4]. Ceruloplasmin has an important role in maintaining iron homeostasis in organism. Showing ferroxidase activity, it facilitates the oxidation of ferrous to ferric iron without the formation of hydroxides and superoxide anion radicals. Additionally, it shows some superoxide dismutase activity and thus it was speculated that it may be an important factor maintaining total plasma antioxidant capacity [5]. Ceruloplasmin is a positive acute phase protein and its serum

concentration increases in response to most inflammatory agents [6]. However, there is no data in the literature evaluating ceruloplasmin concentrations in PF of women with endometriosis.

Haptoglobin is a serum  $\alpha_2$ -glycoprotein produced by hepatocytes in response to inflammation and infection. Thus, it is also classified as a *positive* acute phase protein [7]. Its serum concentration increases even to 500% up to 7 days after inflammatory stimulus and typically normalizes within 2-4 weeks after the inflammatory process resolves [8]. Haptoglobin concentration typically decreases in the course of haemolytic and liver diseases, and during last weeks of pregnancy [9]. Haptoglobin plays an important role in oxidative stress prevention. It binds free hemoglobin with extremely high affinity preventing hydroxyl radicals production [10]. Besides its anti-oxidative capacity, haptoglobin influences the immune system, showing significant inhibitory activity on the biosynthesis of Th2 cytokines [11]. It possesses ability to bind monocytes, macrophages as well as B lymphocytes, granulocytes, NK cells, and CD8 lymphocytes [12]. Cid et al. [13] proved that haptoglobin promotes both endothelial cells growth and blood vessels differentiation, demonstrating direct proangiogenic properties.

There were no differences observed in haptoglobin levels and haptoglobin/protein index in PF of women with endometriosis and healthy controls [14,15]. However, according to Ferrero et al. [16], the haptoglobin  $\beta$  chain (Hp $\beta$ E) concentration is increased in plasma and PF of endometriotic women. Similarly, levels of  $\alpha$ -1B-glycoprotein, an isoform of haptoglobin  $\alpha$  chain, are higher in PF of women with I and II stages of the disease [17].

Sharp et al. [18] identified haptoglobin-like protein showing immunosuppressive and angiogenic properties in human uterine endometrium cell cultures and rats with surgically induced endometriosis. The protein was named ENDO-I (endometriosis protein-I). Further investigations confirmed enhanced ENDO-I production in both eutopic and ectopic endometrium of patients

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suffering from endometriosis when comparing to endometrium of healthy women [19]. Haptoglobin expression was also found in endometrial tissue fragments isolated from PF but the level of the expression did not differ significantly between women with endometriosis and healthy controls [20]. Macrophages isolated from PF of endometriotic patients bind significantly more haptoglobin and secrete markedly more IL-6 than peritoneal macrophages of healthy women [15].

The objective of the study was to assess concentrations of two markers of the acute inflammatory phase proteins, haptoglobin and ceruloplasmin, in PF of women with endometriosis.

## Material and methods

We examined 229 women, aged 15-53, who underwent diagnostic or therapeutic laparoscopy. Clinically and histologically confirmed diagnosis established the following groups: women with endometriosis (E, n=110), and as the reference groups, patients with simple serous (R1, n=78) and dermoid (R2, n=41) ovarian cysts. In each case, endometriosis was staged according to the American Society for Reproductive Medicine classification [21]. The disease was found to be minimal (E1) in 23 cases, mild (E2) in 25 patients, moderate (E3) in 39 women and severe (E4) in 23 cases.

Medical history of the patients and basic clinical examination showed no general chronic diseases, except for the condition, which was the indication for laparoscopy. Mean age of women did not differ significantly between the studied groups. Similarly, no significant difference was found in the phase of menstrual cycle of the time of laparoscopic procedures between women in all study groups.

All patients signed an informed consent and the Lublin Medical University Ethics Committee approval was obtained for the study.

All visible PF was aspirated during laparoscopy from the anterior and posterior cul-de-sacs. Samples were immediately centrifuged, and the supernatants were aspirated and stored at -70°C until analysis. Haptoglobin and ceruloplasmin concentrations in PF were measured using radial immunodiffusion kits (Human Haptoglobin Bindarid, Prod. Code RN058.3 and Human Ceruloplasmin Bindarid, Prod. Code RN045.3; The Binding Site Ltd). After calculation of the assay results, PF concentrations of haptoglobin and ceruloplasmin were expressed in nanograms per milliliter.

All data were tested with the Shapiro-Wilk test for normality. Statistical significance was determined with the Mann-Whitney U and H Kruskal-Wallis tests. Spearman's rank coefficient test was used to assess the relationship between variables. P value less than 0.05 was considered statistically significant. Data are presented as medians (Me), minima (Min), maxima (Max), lower and upper quartiles.

## Results

The concentration of haptoglobin in PF of patients with endometriosis was significantly higher compared to women from both reference groups ( $p < 0.01$ ) (Figure 1). Levels of haptoglobin in PF of women with serous ovarian cysts were similar to those noted in patients with dermoid cysts.

Significantly higher PF haptoglobin level was observed in patients with severe and moderate endometriosis as compared

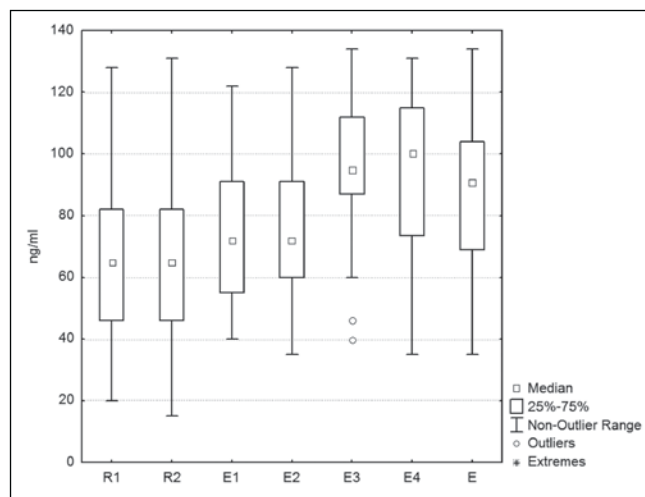


Figure 1. Haptoglobin concentrations in the PF of studied groups.

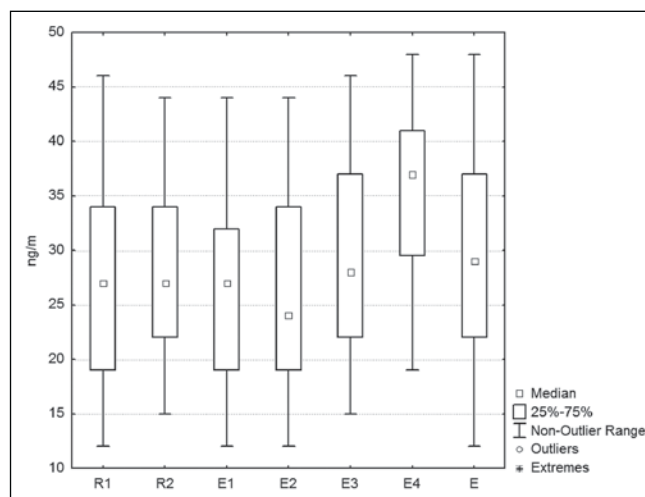


Figure 2. Ceruloplasmin concentrations in the PF of studied groups.

to women from both reference groups. Women with moderate endometriosis had higher peritoneal haptoglobin concentrations compared to patients with stage II endometriosis. No significant differences were found between levels of haptoglobin in PF of women with minimal and mild disease and patients from both reference groups (Table I).

No significant difference in PF ceruloplasmin levels was found between patients with endometriosis and women from reference groups ( $p > 0.8$ ) (Figure 2). PF ceruloplasmin levels did not differ significantly between the reference groups. However, by analyzing concentrations of ceruloplasmin in PF of women with different stages of the disease, it was noted that they are higher in the subgroup of patients with stage IV endometriosis as compared to both reference groups and women with mild endometriosis (Table II.).

## Discussion

Accumulating data suggest that endometriosis is associated with systemic subclinical inflammation. To test the hypothesis of endometriosis-induced pelvic inflammatory process, we inves-

**Table I.** P values for comparisons of haptoglobin PF concentrations between study groups.

Variable: haptoglobin	P values for multiple comparisons (two-sided comparisons) Kruskal-Wallis test					
	R1	R2	E1	E2	E3	E4
R1		NS	NS	NS	<b>0,000</b>	<b>0,001</b>
R2	NS		NS	NS	<b>0,000</b>	<b>0,005</b>
E1	NS	NS		NS	NS	NS
E2	NS	NS	NS		<b>0,022</b>	NS
E3	<b>0,001</b>	<b>0,000</b>	NS	<b>0,022</b>		NS
E4	<b>0,001</b>	<b>0,005</b>	NS	NS	NS	

NS: not significant.

**Table II.** P values for comparisons of ceruloplasmin PF concentrations between study groups.

Variable: ceruloplasmin	P values for multiple comparisons (two-sided comparisons) Kruskal-Wallis test					
	R1	R2	E1	E2	E3	E4
R1		NS	NS	NS	NS	<b>0,010</b>
R2	NS		NS	NS	NS	<b>0,049</b>
E1	NS	NS		NS	NS	NS
E2	NS	NS	NS		NS	<b>0,048</b>
E3	NS	NS	NS	NS		NS
E4	<b>0,010</b>	<b>0,049</b>	NS	<b>0,048</b>	NS	

NS: not significant

No statistically significant correlation was found between PF haptoglobin and ceruloplasmin levels ( $R=0.098$ ;  $p=0.17$ ).

tigated PF levels of haptoglobin and ceruloplasmin in patients with endometriosis. In the study, we demonstrated that the concentration of haptoglobin in PF of women with endometriosis was significantly higher as compared to patients with serous and dermoid ovarian cysts. However, analyzing the results in terms of severity of endometriosis, we found that this difference occurs only in patients with moderate and severe disease. These results are in accordance with the ones gained by Dunselman et al. [14]. Admittedly, they did not show differences in concentrations of haptoglobin in PF between healthy women and patients with endometriosis. However, it is worth mentioning that their work was performed on the group of 25 patients only. The investigators recruited patients with minimal and mild disease, and did not include patients with advanced disease. Thus, it can be assumed that the conclusions they drew may be incomplete and biased. Similarly, Sharpe-Timms et al. [15] demonstrated no significant differences in the concentration of haptoglobin in PF of healthy women and patients with endometriosis. Also in this case, the study group consisted of 11 endometriotic patients only. Five of them had I and II stage of endometriosis, four had III and IV stage, and the ASRM classification was not applied in two patients. Due to the small sample size and the majority of women with minimal and mild disease, the results obtained by the investigators in the studies may not be compatible and consistent with the results gained in the presented clinical trial.

The concentration of haptoglobin increases during inflammation processes. The results obtained in the present trial suggest that in endometriotic patients, an increase in haptoglobin concentration in PF microenvironment may be the result of strong pro-inflammatory stimuli acting intensively only in the advanced stages of the disease. Haptoglobin, by binding free hemoglobin and preventing the superoxide anion radicals formation, plays a significant role in oxidative stress prevention. Higher concentrations of haptoglobin in PF in women with endometriosis can hypothetically accompany the development of the disease. This glycoprotein promotes growth of the endothelium cells and the processes of neovascularization [13]. It therefore cannot be ruled out that the raised concentration of haptoglobin in PF is one of the factors promoting vascularization of the growing endometrial implants.

Concentration of ceruloplasmin in PF of women with endometriosis did not differ significantly between the study and the reference groups. However, detailed analysis showed higher concentrations of ceruloplasmin in PF of women with severe stage of the disease. It is worth mentioning that this observation is pioneering, and no studies investigating the role of ceruloplasmin in PF of endometriotic patients have been published yet. Therefore, we cannot compare and discuss our results with published data. It seems that observed high PF ceruloplasmin concentrations are closely related with the severity of the inflammation process,

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which occurs in the IV clinical stage of endometriosis. Increased concentrations of this glycoprotein in the severe stadium of the disease can prevent activation of the free radical process, by oxidizing the ferrous ions to ferric.

## Conclusion

In conclusion, we have observed higher concentrations of two protein markers of the acute inflammatory phase (haptoglobin and ceruloplasmin) in PF of women with advanced stages of endometriosis. These results support the hypothesis that endometriosis is associated with subclinical inflammation within the peritoneal cavity [22]. However, our findings suggest that in the case of endometriosis only the advanced stages of the disease induce pro-inflammatory stimuli strong enough to cause increase in PF haptoglobin and ceruloplasmin levels.

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## Konflikt interesów:

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## References

1. Gazvani R, Templeton A. Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. *Reproduction*. 2002, 123 (2), 217-226.
2. Harada T, Iwabe T, Terekawa N. Role of cytokines in endometriosis. *Fertil Steril*. 2001, 76 (1), 1-10.
3. Holmberg CG, Laurell CB. Investigation serum copper. II. Isolation of the copper containing protein, and a description of some its properties. *Acta Chemica Scand*. 1948, 2, 550-556.
4. Vassiliev V, Harris ZL, Zatta P. Ceruloplasmin in neurodegenerative diseases. *Brain Res Rev*. 2005, 49 (3), 633-640.
5. Dumoulin MJ, Chahine R, Atanasiu R, [et al.]. Comparative antioxidant and cardioprotective effects of ceruloplasmin, superoxide dismutase and albumin. *Arzneimittelforschung*. 1996, 46 (9), 855-861.
6. Nazifi S, Razavi SM, Esmailnejad Z, [et al.]. Study on acute phase proteins (haptoglobin, serum amyloid A, fibrinogen, and ceruloplasmin) changes and their diagnostic values in bovine tropical theileriosis. *Parasitol Res*. 2009, 105 (1), 41-46.
7. Carter K, Worwood M. Haptoglobin: a review of the major allele frequencies worldwide and their association with diseases. *Int J Laborat Hematol*. 2007, 29 (2), 92-110.
8. Gabay C, Kushner I. Mechanisms of disease: acute-phase proteins and other systemic responses to inflammation. *New J Engl Med*. 2009, 340 (17), 448-454.
9. Piessens MF, Marien G, Stevens E. Decreased haptoglobin levels in respiratory allergy. *Clin Allergy*. 1984, 14 (3), 287-293.
10. Sadrzadeh SM, Graf E, Panter SS, [et al.]. Hemoglobin: A biologic fenton reagent. *J Biol Chem*. 1984, 259 (23), 14354-14356.
11. Arredouani M, Matthijs P, Van Hoeyveld E, [et al.]. Haptoglobin directly affects T cells and suppresses T helper cell type 2 cytokine release. *Immunol*. 2003, 108 (2), 144-151.
12. Hanasaki K, Powell LD, Varki A. Binding of human plasma sialoglycoproteins by the B-cell specific lectin CD22. Selective recognition of immunoglobulin M and haptoglobin. *J Biol Chem*. 1995, 270 (13), 7543-7550.
13. Cid MC, Grant DS, Hoffman GS, [et al.]. Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. *J Clin Invest*. 1993, 91 (3), 977-985.
14. Dunselman GA, Bouckaert PX, Evers JL. The acute-phase response in endometriosis of women. *J Reprod Fertil*. 1988, 83 (2), 803-808.
15. Sharpe-Timms KL, Zimmer R, Ricke EA, [et al.]. Endometriotic haptoglobin binds to peritoneal macrophages and alters their function with endometriosis. *Fertil Steril*. 2002, 78 (4), 810-819.
16. Ferrero S, Gillott DJ, Remorgida V, [et al.]. Haptoglobin beta chain isoforms in the plasma and peritoneal fluid of women with endometriosis. *Fertil Steril*. 2005, 83(5), 1536-1543.
17. Ferrero S, Gillott DJ, Remorgida V, [et al.]. Peritoneal fluid proteome in women with different ASRM stages of endometriosis. *Gynecol Endocrinol*. 2008, 24 (8), 433-441.
18. Sharpe KL, Vernon MW. Polypeptides synthesized and released by rat endometriotic tissue differ from those of the uterine endometrium in culture. *Biol Reprod*. 1993, 48 (6), 1334-1340.
19. Sharpe-Timms KL, Ricke EA, Piva M, [et al.]. Differential expression and localization of de novo synthesized endometriotic haptoglobin in endometrium and endometriotic lesions. *Hum Reprod*. 2000, 15 (10), 2180-2185.
20. Sharpe-Timms KL. Haptoglobin expression by shed endometrial tissue fragments found in peritoneal fluid. *Fertil Steril*. 2005, 84 (1), 22-30.
21. American Society for Reproductive Medicine. Revised American Society for Reproductive Medicine classification of endometriosis. *Fertil Steril*. 1996, 67 (5), 817-821.
22. Polak G, Wertel I, Kwaśniewski W, [et al.]. Udział metabolizmu żelaza oraz stresu oksydacyjnego w patogenezie endometriozy. *Ginekol Pol*. 2013, 84 (1), 62-64.