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Endometriosis is associated with an increased whole-blood thrombogenicity detected by a novel automated microchip flow-chamber system (T-TAS®)

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ABSTRACT

Objectives: Potential thrombotic and antifibrinolytic influence of endometriosis on haemostasis has been recently reported in the literature, as well as increased cardiovascular morbidity in women suffering from the disease. We performed a pilot study to assess the influence of endometriosis on the thrombus formation process under *in vitro* flow conditions.

Material and methods: This study compared women with confirmed endometriosis (n = 23) surgically and control healthy subjects (n = 10). In both groups, the same exclusion criteria were used: a prior episode of thrombosis diagnosed as acquired or inherited thrombophilia, neoplasm, and an uncertain family history of thrombosis. We evaluated the whole blood thrombogenicity using T-TAS® at a shear rate of 240 s-1 (Total-Thrombus Analysis System, Zacros, Japan).

Results: The blood clot formation initiation time (T10) and occlusion time (OT) were significantly shortened in the endometriosis group ($p < 0.05$). The area under the curve (AUC30) of blood clot time formation values (BCTF) was substantially higher in the patients suffering from a disease ($p = 0.03$). An increase in AUC (TTAS) values by 100 increases the risk of developing endometriosis by 1.56-fold [adjusted OR = 1.56 ($p = 0.01$); (95% CI: 1.10–2.18)]. Inflammatory markers (neutrophil-to-lymphocyte ratio (NLR), and the leucocyte, neutrophil, basophil, and neutrophil concentrations) were also substantially higher in the endometriosis group ($p < 0.05$).

Conclusions: The alteration of the T-TAS® and NLR values supports the thesis of a shift of the equilibrium towards thrombosis in women who have endometriosis. This phenomenon links to a state of chronic inflammation. It is detectable using a novel system for the quantitative assessment of the platelet thrombus formation process under flow conditions *in vitro*.

Key words: endometriosis; thrombosis; prothrombotic state

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INTRODUCTION

Endometriosis is a disease defined by the occurrence of endometrial glands and stroma outside the uterine cavity [1]. It remains a relatively prevalent gynaecological disorder. That disease affects from 6% to 10% of women of reproductive age in the United States of America [2].

Despite this, the aetiology of the disorder is still elusive [3]. A growing body of scientific evidence has recently been linking endometriosis with an immunologic dysfunction, metabolic changes, the atherogenic lipid profile, oxidative stress as well as with chronic systemic inflammation [4, 5]. Moreover, this disease may be a significant risk factor for major chronic diseases, although the underlying mechanism is not yet fully understood [6].

In contemporary research, an association of endometriosis with many common systemic comorbidities, such as cancers, allergy, autoimmune and cardiovascular diseases have been found [6–8]. Coronary heart disease (CHD) remains an example of arterial thrombosis, and in a large prospective study, laparoscopically confirmed endometriosis was associated with an increased risk of CHD episodes. That association was more influential in the younger group of women who have endometriosis. Moreover, hysterectomy/oophorectomy procedures and hormonal therapy increase the risk of CHD; however, those can only partially explain the link of CHD with endometriosis [9–11]. After adjustments for potential confounders, the relative risk of CHD in Endometriosis is 1.62-fold increased, in comparison to non-endometriosis women [6].

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Various inflammatory factors such as intracellular adhesion molecule 1 (ICAM-1), interleukin-1 and interleukin-6 (IL-1 and IL-6), tumour necrosis factor-alpha α (TNF- α) and vascular endothelial growth factor (VEGF) and vitamin D polymorphism alteration have been described in endometriotic patients [12–16]. The systemic inflammatory response results in changes in the white blood cells (WBC) and the coexistence of relative lymphocytopenia and neutrophilia has been observed [17, 18]. Thus, the increased ratio of neutrophil-to-lymphocyte (NRL) in endometriosis women strongly suggests the occurrence of chronic inflammation in this group of patients [19]. In endometriosis, an increased level of activated macrophages and increased levels of the cytokines provides a local microenvironment suitable for the initiation and growth of endometriotic lesions [20].

A growing body of research supports the thesis that inflammation and haemostasis processes interact substantially with each other [21]. The coagulation modulates the inflammation, and the inflammation activates the coagulation cascade [22, 23]. The inflammatory response enhances and promotes the initiation of coagulation, increasing the risk of organ dysfunction and microvascular thrombosis [24, 25].

In endometriosis patients, the haemostasis and coagulation assay values are altered. In 2015, Wu et al. [21], proved that in women with endometriosis, the APTT (activated partial thromboplastin time), and TT (thrombin time) are shortened while the fibrinogen concentration is elevated. Moreover, in their blood, a higher percentage of circulating degranulated platelets was found [21]. Several studies have demonstrated that those changes are involved in the development of endometriosis. Endometriosis women appear to remain in a hypercoagulable and hypofibrinolytic state due to platelet aggregation in endometriotic lesions. It provides a potential rationale for the use of anticoagulants to treat endometriosis [26].

Platelet size is an essential marker of platelet function. Mean platelet volume (MPV) remains an indicator of platelet activation, and increased values link to platelet hyperreactivity. Thus, MPV is also an important biological variable predicting the risk of arterial micro thrombotic episodes such as vascular complications in diabetes mellitus. Liu et al. [27], showed an association of MPV with the severity of diabetic retinopathy.

There are numerous methods for assessing haemostasis. However, screening coagulation assays are not sensitive enough to detect hypercoagulation adequately and thus remain unable to evaluate thrombotic risk [28]. Efforts have been made to use global, integral assays to mimic and reflect aspects of haemostasis *in vitro*. An example of such is the Total Thrombus-formation Analysis System (T-TAS®). This system enables the evaluation of the *in vitro* thrombus formation process under flow conditions. (T-TAS®) is an innovative microchip flow-chamber system enabling quantitative assessment of the platelet thrombus formation process. This assay represents noteworthy improvements over conventional platelet function assays. The fact that T-TAS® is sensitive not only to platelet aggregation but also detects alterations occurring in the fibrinolytic system is very important considering the aim of our study [29].

Many previous studies have demonstrated the influence of endometriosis on some coagulation assays, but still, conventional coagulation assays are often not sensitive enough to detect hypercoagulation and are unable to evaluate the risk of developing thrombosis. To date, there has been no research study assessing the influence of endometriosis on the process of thrombus formation under flow conditions *in vitro*.

Our study aimed to evaluate the whole blood thrombogenicity in women who have endometriosis to assess in this pilot study the occurrence of any alteration of haemostasis in this group of women.

MATERIAL AND METHODS

Subjects

We conducted the study in the Department of Reproduction at the Poznan University of Medical Sciences from December 2017 until March 2018. The analysis included 23 patients with surgically confirmed endometriosis as well as 10 healthy women, who formed the control group.

We collected the data of patients who were referred to the gynaecological ward due to a suspicion of endometriosis. All the patients from the endometriosis group suffered from at least one of the clinical symptoms and signs of the disease such as dysmenorrhea, dysuria, dyspareunia, dyschezia, subfertility, or infertility.

We took a detailed history which included a list of varied gynaecological items from every subject in the control group. All women from this group presented regular monthly menses with standard length and denied using any medications, including oral contraceptives, in the previous 12 months.

Every woman from the control group denied the occurrence of symptoms such as ever having been diagnosed or having a suspected diagnosis of endometriosis.

Moreover, in both groups, the same additional exclusion criteria were used to avoid significant confounding factors, such as an episode of venous or arterial thrombosis in the prior history, diagnosed acquired or inherited thrombophilia, neoplasm and an uncertain family history of thrombosis, pregnancy, hypertension, smoking and the occurrence of any vascular disease.

Methods

Blood samples for all tests in both groups were taken in the follicular phase of the menstrual cycle. In patients undergoing surgical procedures, blood samples were taken a day before the scheduled surgery.

Standard comparative laboratory findings

All biochemical tests were performed in the accredited laboratory of the University hospital (Central Laboratory, The Obstetrics and Gynecology Teaching Hospital of Poznan University of Medical Sciences), which holds an ISO 9000 certificate of quality management. We collected blood samples for standard conventional laboratory tests at the same time in a fasting state from the antecubital veins.

After being drawn, the blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Ethylenediaminetetraacetic acid was used as an anticoagulant to prevent clotting. The samples we immediately transported to the laboratory. Testing was performed with the use of an automated flow cytometry haematology analyser (Roche Diagnostics®).

T-TAS® findings

Evaluation of whole blood thrombogenicity (assessment of the formation of a thrombus under flow conditions) was assessed immediately after taking the blood samples using T-TAS® at a shear rate of 240 s-1 (Total Thrombus Analysis System, Fujimori Kogyo, Zacros, Japan, AR-chip) equipped with AR microchip and thrombogenic surfaces (collagen with thromboplastin).

For each test, the patient blood samples were analysed for thrombus formation area under a flow pressure curve for 30 min (AUC30 indicates the area under the flow pressure curve for the 30 min.), time of blood clot formation initiation (T10) and OT or T80 (an occlusion time — time of complete thrombus formation inside the AR-chip). T80-T10 — time of growth of the thrombus on AR chip (duration between the time of initiation of formation of the blood clot, and occlusion time). T10 is defined as the time of the onset of the formation of a thrombus. It resembles the duration of the flow pressure from baseline values to the ten kPa pressure values. It is related with the partial occlusion of the microcapillary. T80 (OT) represents the time of complete occlusion of the microcapillary, occurring with a pressure of 80kPa. T80-T10 is the interval between T10 and OT or T80 and represents the rate of growth of a thrombus. AUC30 remains an area under the 80 kPa flow pressure curve for 30 min after the start of the assay in the AR chip.

Statistical analysis

Statistical analysis was performed using PQ STAT® statistical software. For normality, we checked the data using the Shapiro-Wilk test, and then appropriate parametric or nonparametric tests were used to assess the differences between the parameters across the two groups. For parametric, independent samples, the t-Test for independent values and the nonparametric Mann-Whitney U test were used. We used Multivariate logistic regression to obtain the OR values. Due to the limited numbers of participants in the analysed groups, in multivariate logistic regression, the use of only one confounder (BMI) was plausible.

The values of the variables are presented as mean ± one standard deviation or median (interquartile range) appropriate to the use of nonparametric and parametric, respectively. A p value of < 0.05 was considered statistically significant.

The study complies with the principles of the Declaration of Helsinki. The Ethics Committee approved the study protocol of the Poznan University of Medical Sciences (approval number 300/17, 2/3/2017).

We took informed written consent from all patients.

RESULTS

In this pilot study, we aimed to compare the whole blood thrombus formation process under flow conditions in endometriosis and healthy individuals. Beyond this, we performed conventional standard laboratory investigations in all groups.

Total thrombus analysis system results

The analysis carried out with the use of T-TAS® showed that in women with endometriosis, the initiation of thrombus formation, and the total clot formation time inside the AR-chip occurred significantly faster compared to the control group. The T10 and OT values were significantly shorter between groups ($p = 0.008$; $p = 0.003$, respectively).

The area under the flow pressure curve for 30 min (AUC) values was significantly higher in the endometriosis group compared to the controls ($p = 0.009$).

Due to the limited number of participants in the analysed groups, in multivariate logistic regression, the use of only one confounder (BMI) was plausible. After analysis adjusted for BMI, a link between an increase by 100 in the AUC-30 values, and a 1.56-fold increased risk of endometriosis was demonstrated, adjusted OR = 1.56 [$p = 0.01$; 95% confidence interval (CI): 1.10–2.18].

Standard laboratory investigations

We observed differences in the complete blood count (CBC) values in the study and control groups. In the endometriosis group, lower erythrocytes [red blood cells (RBC)], haemoglobin (HGB) concentrations, and the haematocrit value were observed (respectively: $p = 0.02$, $p = 0.0008$; $p = 0.03$). The occurrence of those differences is caused mainly by the heavy menstrual bleeding reported by patients from the study group.

Inflammation status analysis revealed substantially higher leucocytes [white blood cells (WBC)] ($p = 0.002$) as well as neutrophil ($p = 0.00002$) and basophils($p = 0.0008$) concentrations in the study group. Additionally, we observed a statistically lower eosinophils concentration in the endometriosis group ($p = 0.003$).

The neutrophil-to-lymphocyte ratio (NLR), which is as a marker of subclinical inflammation, was almost threefold higher in the endometriosis group compared to the healthy controls. This difference across those two groups was statistically significant ($p = 0.0001$).

Agranulocytes: the lymphocyte and monocyte concentrations did not differ across the groups.

Additionally, there were no differences observed in the PLR (Platelet-to-lymphocyte ratio) between the groups in our study. PLR assessment is a novel index reflecting a systemic inflammatory burden that combines the prognostic values of an individual's platelet and lymphocyte counts.

Our study group comprised of 23 surgically confirmed endometriosis patients. Revised American Society for Reproductive Medicine classification (rASRM) of endometriosis to classify lesions during surgical procedures has been used. With I and II stage of endometriosis were diagnosed 5 and with III and IV 18 patients, respectively. We used only rASRM. Due to non-parametric data median with 1st (lower) and 3rd (upper) quartile were reported in Table 1. (4; 3–4).

Values and appropriate p values are shown in Table 1.

DISCUSSION

In the present study, we investigated the prothrombotic influence of endometriosis using a novel automated microchip flow chamber system for the quantitative analysis of thrombus formation: Total Thrombus-formation Analysis System (T-TAS®). To date, there has been no research study assessing the influence of endometriosis on the process of thrombus formation using this system, and no such data has been obtained in women who have endometriosis yet.

T-TAS® enables the assessment of the whole blood thrombogenicity [29]. It was designed to evaluate the prophylaxis of various antiplatelet drugs against thrombosis, based on the phenomenon that platelets remain crucial to initiating thrombus formation. This system further allows an evaluation of the *in vitro* thrombus formation process under flow conditions. It is also useful in assessing the clot formation process in haemostasis alterations such as factor VIII deficiency (mouse model) [30]. Previous research on a T-TAS® showed that the system might be useful in assessing the effects of antithrombotic agents [29, 31]. Before this study, the impact of endometriosis on haemostasis using T-TAS® had not been analysed.

Increased whole-blood thrombogenicity has been demonstrated in the Endometriosis group.

Augmented blood clotting in those patients is not recognisable using a standard test such as mean platelet value (MPV). Moreover, the platelet count values were not different in the study and control groups. Therefore, our data strongly suggest that T-TAS® might be a sensitive diagnostic tool to detect a prothrombotic state occurred in women who have endometriosis, however, our findings need further elucidation in research on significantly more extensive data sets to be confirmed.

There is a considerable body of evidence that endometriosis predisposes to alter the coagulation status in women [32]. Thus, our findings remain consistent with the literature. We support the thesis of a shift towards thrombosis in the haemostasis milieu in that group of patients. Ding et al. [33], showed a higher platelet aggregation rate, fibrinogen concentration, platelet activation rate, elevated plasma D-dimer, plasma soluble P-selectin, prothrombin fragment 1 + 2 (F1 + 2), fibrin degradation products and shortened thrombin time. In a study conducted by Qinjiao et al. [21], APTT and TT were shortened, and elevated fibrinogen levels were documented. The authors of this study suggested the possibility of a therapeutic role of anticoagulant agents and their potential future use in the treatment of endometriosis. There are numerous studies with a suggestive role in the activation of the coagulation cascade in endometriosis. The potential role of a tissue factor, in the etiopathogenesis of the disease, has been demonstrated as an angiogenic parameter [34].

In this study, we documented that using the T-TAS® system. We can detect a significant difference in the time of initiation, elongation and the total time of thrombus formation in healthy vs endometriosis subjects. Moreover, T-TAS® not only detects differences in whole-blood thrombogenicity mediated by platelet hyperreactivity but is also sensitive to alterations occurring in the fibrinolytic system. Our results support the thesis that endometriosis is associated with hyperfibrinolysis, and thus they remain consistent with prior research studies on this topic. The fibrinolytic system plays a significant role in maintaining homeostasis. It affects not only the haemostatic balance but also tumour invasion, tissue remodelling, reproduction and angiogenesis [35]. Plasminogen activator inhibitor-1 (PAI-1) is a significant inhibitor of the fibrinolytic system [36]. A growing body of evidence indicates that increased levels of PAI-1 mediate tumour metastasis, diabetes, cardiovascular and reproductive diseases [37–40]. Bedaiwy et al. [15], showed that endometriosis is associated with hyperfibrinolysis due to the more frequent occurrence of the 4G allele of the *PAI-1* gene in surgically confirmed endometriosis patients [15]. There is, however, a dispute in the link between *PAI-1* polymorphic variants and susceptibility to endometriosis [41, 42]. Hypofibrinolysis promotes a fibrin matrix, thus supporting

Table 1. Characteristics of the studied groups

Variable	Control*	SD/ Lower quartile*	Upper quartile*	Endometriosis*	SD/ Lower quartile*	Upper quartile*	p**
	n = 10			n = 23			
BMI [kg/m^2]	21.55	20.56	23.83	22.00	19.76	23.55	0.8
Endometriosis stage [rASRM]	N/A			4 (I = 2; II = 3; III = 5; IV = 13)	3	4	–
Comparative laboratory investigations							
Erythrocytes (RBC) [G/L]	4.45	4.33	4.65	4.07	3.99	4.47	0.02
RDW (Red cell distribution width) [%]	12.95	12.38	13.35	13.10	12.25	13.45	0.16
MCV [fL]	85.26	3.08		85.11	5.34		0.96
MCH [fmol]	1.92	1.78	1.94	1.81	1.70	1.86	0.07
MCHC [mmol/L]	21.76	0.80		20.72	0.76		0.001
HGB [mmol/L]	8.32	0.61		7.44	0.63		0.0008
HCT [L/L]	0.38	0.02		0.36	0.03		0.03
Leukocytes (WBC) [G/L]	6.18	4.82	6.55	9.88	6.22	12.02	0.002
Neutrophiles [G/L]	3.23	1.03		7.91	4.06		0.00002
Eozynophyles [G/L]	0.10	0.09	0.16	0.04	0.02	0.085	0.003
Basophyles [G/L]	0.02	0.02	0.02	0.04	0.03	0.045	0.0008
Lymphocytes [G/L]	1.88	0.68		1.66	0.55		0.33
Monocytes [G/L]	0.49	0.44	0.53	0.67	0.42	0.82	0.16
Platelets [G/L]	270.50	206.00	279.00	255.00	219.50	291	0.52
MPV [fl]	11.12	0.67		10.56	0.67		0.03
Platelet-to-lymphocyte ratio (PLR)	133.27	119.51	181.01	164.36	125.07	203.99	0.36
Neutrophil-to-lymphocyte ratio (NLR)	1.66	1.41	2.11	4.79	3.11	6.34	0.0001
Endometriosis stage	N/A			4	3	4	-
Comparative T-TAS® findings							
T10 [s]	639.40	284.07		325.70	165.45		0.008
AUC30	1468.65	398.35		1893.40	233.68		0.009
OT [s]	800.50	599.50	926.50	385.00	328.50	511	0.003

*Mean with SD (standard deprivation) are presented for parametric data. Median with 1st (lower) and 3rd (upper) quartile are reported for non-parametric data; **For parametric: independent-samples t-Test and for nonparametric U Mann-Whitney test were used; Note: Statistically significant p values are indicated in bold; BMI — body mass index; N/A — not applicable; RBC — red blood cells; MCV — mean corpusculat volume; MCH — mean corpuscular hemoglobin; MCHC — mean corpuscular hemoglobin concentration; HGB — haemoglobin; HCT — hematocrit; WBC — white blood cells; MPV — mean platelet volume; T10 — time of blood clot formation initiation; AUC30 — area under the curve; OT — occlusion time

the initiation of endometrial lesions in the peritoneal cavity caused by retrograde menstruation [15].

Moreover, in peritoneal fluid from patients with endometriosis, increased PAI-1 antigen links with an increased risk of peritoneal adhesions [42]. PAI-1 regulates tumour cell migration and the mechanism of endometriotic cell invasion [43, 44]. This notion could be potentially a piece of the explanation of why some women develop the endometriosis phenotype while others do not [15]. *PAI-1* 4G/5G links with endometriosis-associated infertility [45]. *PAI-1* antigenic levels were higher in ovarian endometriomas than in a normal eutopic endometrium [46]. In *in vitro* studies, endometrial stromal tissues sampled from women with endometriosis released higher PAI-1 antigen levels than tissues derived from a healthy control group. The higher release of PAI-1 might be an essential phenomenon in the invasive growth process which occurs in endometriotic lesion formation [43].

The chronic inflammatory process related to the increase of cytokines and chemokines occurring in patients with endometriosis has been well demonstrated in previous research [47].

In our study, we showed a higher NLR (neutrophil-to-lymphocyte ratio) in women with endometriosis. Thus, our results remain consistent with prior research. Tokmak et al. [48], showed higher NLR values in endometriosis patients and stated that the combination of Ca-125 levels and NLR

values improves diagnostic accuracy in detecting endometriomas. Cho. et al. [49], evidenced that NLR levels can discriminate endometriosis patients from controls with 83.9% specificity when combined with other markers such as Ca-125. Our study fails to indicate MPV as a marker of platelet hyperreactivity in women with endometriosis.

Our study group comprised of patients, who underwent surgery due to endometriosis. We have been struggling with defining the reasons for differences in CBC values across the analysed groups, therefore we have stated a hypothesis that those may be related with menstrual disorders, which are more plausible in women suffered from a disease. In authors' opinion more studies are required to assess eventually plausible linkage between CBC and whole blood thrombogenicity measured by T-TTAS® values alterations in women affected by endometriosis. Using this limited data, it is very difficult to answer the question whether CBC alteration have affected T-TAS® values unequivocally; what may be considered as a limitation of our study.

Summarising, our findings strongly support the thesis that endometriosis triggers a shift toward a prothrombotic and antifibrinolytic state, and this phenomenon can be detectable using a novel microchip flow-chamber system for quantitative assessment of the platelet thrombus formation process under flow conditions *in vitro*. The results obtained by this system have shown that each increase in AUC (T-TAS®) values by 100 increases the risk of developing endometriosis by 1.56-fold.

Due to the limited data and limited sample size of our study, the role of T-TAS® alterations in endometriosis patients needs to further elucidation with much larger data sets in order to confirm our essential findings.

CONCLUSIONS

The potential thrombotic and antifibrinolytic influence of endometriosis on haemostasis, as well as increased cardiovascular morbidity in women suffering from a disease, have recently been reported in the literature.

T-TAS® (total thrombus formation analysis) is a useful diagnostic tool allowing the assessment of whole blood thrombogenicity. In our study, the (T-TAS®) results substantially differed in the endometriosis and healthy control groups. Blood clot formation initiation time and occlusion time were significantly shortened in endometriosis, which may indicate the prothrombotic state of this disease. The alteration of (T-TAS®), the NLR (neutrophil-to-lymphocyte ratio) and the granulocytes concentration values in endometriosis support the thesis of a shift of the equilibrium towards thrombosis occurring in patients suffering from the disease, as well as the plausible linkage of this phenomenon with the chronic inflammatory status also found in this group of patients.

Conflict of interest

All authors declare no conflict of interest.

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