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Hemorheological and hemostatic alterations in celiac disease and inflammatory bowel disease in comparison with non-celiac, non-IBD subjects (HERMES): A case-control study protocol

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Abstract

Introduction

Hemorheological and hemostatic changes predispose to the development of arterial and venous thrombotic events; however, limited information is available on the status of these changes in celiac disease (CeD) and inflammatory bowel disease (IBD). In this study, we aim to describe the hemorheological and hemostatic profiles of CeD and IBD patients in a Hungarian cohort of patients to investigate whether any alterations contribute to elevated thrombotic risk.

Methods and analysis

This is a case-control study involving newly diagnosed and followed CeD and IBD patients with age- and sex-matched non-CeD, non-IBD subjects with an allocation ratio of 1:1:1.

After informed consent is obtained, a detailed medical history will be collected, including venous and arterial thrombotic risk factors and medications. Symptoms in CeD patients will be assessed with the Gastrointestinal Symptoms Rating Scale, and disease activity in IBD patients will be determined by calculating the Mayo Score or Crohn's Disease Activity Index. A single trained dietitian will assess dietary adherence among CeD patients with a thorough interview together with a measurement of self-reported adherence, dietary knowledge, and urine analysis (detection of gluten immunogenic peptides). In addition to routine laboratory parameters, hemorheological (i.e., erythrocyte deformability and aggregation, viscosity of whole blood and plasma) and hemostatic parameters (e.g., protein C, protein S, and antithrombin) with immunological indicators (i.e., celiac-specific serology and antiphospholipid antibodies) will be measured from venous blood for every participant.

Primary and secondary outcomes will be hemorheological and hemostatic parameters, respectively. Univariate and multivariate statistics will be used to compare CeD and IBD patients to control subjects. Subgroup analysis will be performed by disease activity.

Ethics and dissemination

The study was approved by the Regional and Local Research Ethics Committee, University of Pécs (Ref No 6917). Findings will be disseminated at research conferences and in peer-reviewed journals.

Trial registration

ISRCTN49677481.

Key words: celiac disease, inflammatory bowel disease, hemorheology, thrombosis

Strengths and limitations of this study

- Immune-mediated bowel diseases are associated with an increased risk of arterial and venous thrombosis, but specific hemorheological and hemostatic alterations are understudied in celiac disease and incomplete in inflammatory bowel disease.
- This case-control study prospectively recruits newly diagnosed and followed-up cases of celiac disease and inflammatory bowel disease with age- and sex-matched controls (the allocation ratio will be 1:1:1, respectively) to investigate clinical and laboratory alterations predisposing to thrombosis.
- Laboratory tests include the measurement of hemorheological (i.e., erythrocyte aggregation and deformability, plasma and whole blood viscosity), hemostatic parameters (e.g., levels of fibrinogen, prothrombin time, protein C, protein S, and antithrombin), and immunological indicators (e.g., celiac-specific serology and antiphospholipid antibodies).
- Patients will be divided by disease activity into active and inactive.
- Results should be interpreted with caution due to the single-centre nature and case-control design of the study.

INTRODUCTION

Immune-mediated disorders may affect 5–7% of the population.[1] These disorders frequently share pathways in pathogenesis as well as organ manifestations. Celiac disease (CeD) and inflammatory bowel disease (IBD) are systemic immune-mediated disorders, primarily affecting the intestines.[2] Both are significant contributors to the load on gastroenterological out-patient clinics due to various diagnostic issues and lifelong follow-up.[3]

CeD and IBD

Global prevalence of biopsy-confirmed CeD is around 0.8%.[4] Genetic (HLADQ2 or DQ8 haplotypes) and environmental factors (ingestion of gluten) play a crucial role in disease development and during the course of the disease. Strict adherence to a lifelong gluten-free diet (GFD) results in symptom relief and a significant reduction in disease complications.[5]

IBDs are less frequent entities: the increasing prevalence of ulcerative colitis and Crohn's disease may reach 0.5% and 0.3% in Europe, respectively.[6, 7] In addition to genetic vulnerability, environmental factors and immune dysregulation are important contributors to disease pathogenesis.[8] Although the pharmacological approach has been improved significantly, treatment imposes a great burden on patients as well as on the healthcare system.[7, 9]

Thrombosis and immune-mediated disorders

Immune-mediated disorders are often characterized by an increased risk of venous and arterial thrombotic events.[10-14] The importance of these events is highlighted by the fact that myocardial infarction, stroke, and venous thrombosis (deep venous thrombosis and pulmonary embolism) are the three most common life-threatening cardiovascular disorders.[15] Patients with atherosclerotic complications carry an increased risk of venous thrombosis, and, conversely, venous thrombosis predisposes to the development of atherosclerotic complications.[16-18] Mechanisms of thrombophilia in immune-mediated disorders are complex, and acquired factors seem important.[19]

Clinical presentation of CeD-associated hypercoagulability includes a wide variety of thrombosis at venous sites, pulmonary embolism, atheroembolism (stroke), and obstetric complications.[20] The multifactorial etiology of thrombosis may embrace the interplay of malabsorption (vitamin and mineral deficiencies, e.g., vitamin B_{12} and K deficiency), thrombophilic autoantibodies (anti-tissue transglutaminase (tTG) and antiphospholipid antibodies), hyperhomocysteinemia, endothelial dysfunction, accelerated atherosclerosis, thrombocyte dysfunction, and genetics.[20-24] Immune-mediated comorbidities ('autoimmune traits'), such as antiphospholipid syndrome, may contribute to the elevated thrombotic risk as well.[24] In addition, ingestion of trace amounts of gluten may maintain a continuous pro-inflammatory response.[25]

IBD is associated with venous thrombosis and pulmonary embolism as well as with the cardiovascular consequences of atherosclerosis, i.e., stroke and myocardial infarction.[26, 27] The increased risk of thrombosis in IBD can be attributed to immobilization, surgical interventions, glucocorticoid therapy, vitamin deficiencies, hyperhomocysteinemia, and chronic inflammation alone or in conjunction with the factors above.[28, 29] In the case of IBD, disease activity may be a crucial determinant of thrombotic risk.[27]

Thrombosis in hemorheological and hemostatic aspects

Rheological properties of blood can be described by specific laboratory measures, including plasma viscosity, blood viscosity, erythrocyte deformability, and erythrocyte aggregability. An altered hemorheological profile contributes to the development of arterial thrombotic events, such as myocardial infarction and stroke.[30-33] In addition, red blood cells and fibrinogen are suspected to be involved in the formation of venous thrombi ('red clots').[34]

Epidemiological studies indicate that arterial and venous coagulopathies are frequently associated with altered levels or function of pro- and anticoagulant proteins (including antithrombin, protein C, and protein S), altered activity of clotting factors, abnormal thrombin generation, and endothelial damage.[35]

Reports indicate that immune-mediated disorders may be associated with hemorheological[36-38] and hemostatic changes[39-41], thereby contributing to the increased risk of thrombotic events.

Prothrombotic hemorheological and hemostatic changes in CeD and IBD

No studies have assessed the hemorheological changes in CeD. There are sporadic reports on activity-dependent prothrombotic hemorheological changes in IBD. However, while individual studies have focused on single outcomes of laboratory parameters, none of them have assessed the complete hemorheological profile of patients.[42-45]

There has only been one retrospective publication that examined the link between CeD and hemostatic alterations in a small cohort of patients: sporadic cases of protein C and protein S deficiency (due to vitamin K malabsorption), hyperhomocysteinemia, and antiphospholipid antibodies were identified.[22] In IBD patients, a significant decline in anticoagulant mechanism is well-established.[46-49]

Scope and objectives

No studies have assessed hemorheological and hemostatic parameters within a study to provide an overall view of thrombotic risk. Since our knowledge of hemorheological and hemostatic changes is limited in CeD and IBD, this study aims to carry out a comprehensive evaluation of venous and arterial prothrombotic alterations in these pro-inflammatory diseases in a Hungarian cohort of patients.

1. Primary objective

- to assess the hemorheological profile of CeD and IBD patients, compared to non-CeD, non-IBD subjects

2. Secondary objective

- to assess the hemostatic profile of CeD and IBD patients, compared to non-CeD, non-IBD subjects

Our results can contribute to expanding our knowledge on the prothrombotic pathophysiological alteration in CeD and IBD, thereby providing the basis for future research on the indications of thromboprophylaxis under special prothrombotic circumstances, such as hospitalization, pregnancy, or immobilization.

METHODS AND ANALYSIS

Design

This is a case-control study with prospective recruitment of CeD and IBD patients with non-CeD, non-IBD control subjects. The study does not change the routine management of subjects included (for the World Health Organization checklist, see Table 1). The study protocol was planned in accordance with the SPIRIT 2013 Statement.[50]

Table 1. World Health Organization checklist

Data category	Information
Primary registry and trial identifying number	ISRCTN49677481
Date of registration in primary registry	05/03/2018
Secondary identifying numbers	None
Source(s) of monetary or material support	University of Pécs Medical School; Momentum Grant from the Hungarian Academy of Sciences (LP2014-10/2014); Highly Cited Publication Grant (KH 125678) from the National Research Development and Innovation Office; GINOP 2.3.2-15-2016-00048 Stay Alive and EFOP 3.6.2-16-2017-00006 Live Longer; Translational Medicine Foundation; and New National Excellence Programme, Ministry of Human Capacities (ÚNKP-17-3-II).
Primary sponsor	None
Secondary sponsor(s)	None
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Public title	Investigation of hemorheological and hemostatic alterations in celiac disease and inflammatory bowel disease in comparison with healthy subjects: A case-control study (HERMES)
Scientific title	Hemorheological and hemostatic alterations in celiac disease and inflammatory bowel disease in comparison with non-celiac, non-IBD subjects: A case-control study (HERMES)
Countries of recruitment	Hungary

Health condition(s) or problem(s) studied	Celiac disease and inflammatory bowel disease
Intervention(s)	Questionnaires (thrombophilia, dietary adherence, disease activity), urine collection (dietary adherence - urine-gluten immunogenic peptide detection), blood collection (hemorheological, hemostatic, and immunological tests complemented with routine laboratory panel)
Key inclusion and exclusion criteria	Inclusion criteria: adult patients (≥18 years of age) suffering from newly diagnosed or treated celiac disease (by ESPHGAN and ACG guidelines), or from inflammatory bowel disease (by ECCO guidelines), and non-celiac, non-IBD subjects
	Exclusion criteria: chronic diseases (chronic kidney diseases, liver cirrhosis, heart failure, active malignant diseases), acute diseases within 2 weeks of inclusion
Study type	Observational
Date of first enrolment	30/5/2018
Target sample size	First phase: 50 celiac and 50 IBD patients plus control (1–3 for each patient). Second phase: target number is determined by power calculation.
Recruitment status	Ongoing
Primary outcome(s)	Hemorheological test results
Key secondary outcomes	Hemostatic test results

Trial organization and steering committee

The Centre for Translational Medicine at the University of Pécs, which was established to advance medical research in gastroenterology, is the coordinator and designer of the HERMES study. The centre is experienced in running investigator-initiated clinical trials.[51] A steering committee will be set up to supervise the entire study process. The Principal Investigator (JB) and the Trial Coordinator (ZS) are responsible for organizing patient recruitment, data collection, sample collection, shipping, and storage, biochemical analysis, and the publication of study results.

Population and eligibility

We will include CeD patients, IBD patients, and non-CeD, non-IBD control subjects. Eligibility criteria will be as follows:

a. Inclusion criteria (applies to all subjects)

- Blood collection must be indicated with medical conditions
- Signed informed consent

b. Inclusion criteria (applies to specific cohorts of patients)

- CeD patients: newly diagnosed or followed patients (with or without adhering to GFD) aged ≥18 years; the establishment of a diagnosis should meet the current guidelines (ESPHGAN, ACG).[5, 52]
- IBD patients: newly diagnosed or followed-up patients (with active or remitting disease) aged ≥18 years (not following GFD); the establishment of a diagnosis should meet the current guidelines (ECCO).[53, 54]
- Non-CeD, non-IBD control subjects: individuals aged ≥18 years (not following GFD) in whom CeD and IBD can be excluded according to the recent guidelines. [5, 52-54]

c. Exclusion criteria (applies to all subjects)

- Chronic conditions:
  - Estimated glomerular filtration rate calculated with CKD-EPI formula is <60ml/min/1.73m^2 (CKD3 or more severe kidney failure)
  - Liver cirrhosis in Child–Pugh B–C
  - Heart failure (NYHA III–IV)
  - Active malignant diseases
- Any acute diseases or invasive procedures within two weeks of recruitment (e.g., systemic infection, surgery, or major trauma)
- Pregnancy
- Patients unable to understand the essentials of the informed consent

Flow and timing

All subjects at our academic hospital for a planned check-up or referred to the center for diagnostic purposes will be recruited consecutively. The place of recruitment will be the Division of Gastroenterology, First Department of Medicine, University of Pécs Medical School. This tertiary centre provides professional gastroenterological care for about 300,000 inhabitants in Baranya County, Hungary.

Recruitment of the study population will be managed in two phases (see 'Target number of patient' section), with the expected recruiting period being between May 2018 and May 2019 (covering one year). Table 2 shows the timeline of the study. Patients will be provided with an information sheet and must provide written consent before sampling. Informed consent will be obtained by personnel with a medical degree. Participants may withdraw from the study for any reason at any time. Consent forms and other related documents will be accessible at https://tm-centre.org.

Table 2. Schedule for the study

TIME POINT	Study period				
	Enrolment	Allocation	Post-allocation		
	-1 hour	0	+1 hour	+1.5 hour	+2 hour
ENROLMENT:					
Eligibility screen	x				
Informed consent	x				
Allocation		x			
INTERVENTION:					
Interview and questionnaire			x		
Urine collection				x	
Blood collection				x	
ASSESSMENT					
Symptom scores and disease activity			x		
Thrombophilia questionnaire			x		
Dietary adherence			x		
Blood analysis[#]					x
Urine analysis[#]					=>[*]

[*]samples will be deep frozen until all participants have been recruited
[#]after analysis, blood and urine residues will be stored in the biobank

Patients will be monitored by our professional data management team throughout the entire process of data and biological sample collection to ensure perfect adherence to protocol. Written feedback will be provided to patients on the results of the laboratory tests and dietary evaluation. If findings indicate, patients will be referred to their general practitioners or a specialist for further investigation and management.

Measurements

All samples will be collected and questionnaires will be administered within two hours after allocation. Actions for each group are defined and listed in Table 3.

Table 3. Actions within study

	CeD patients	IBD patients	Control subjects
Thrombophilia questionnaire	+	+	+
GSRS	+	-	+
Dietary interview and GFD adherence tests	+	-	+
Mayo Score/CDAI	-	+	+

Urine GIP detection	+	-	+
Laboratory measures			
routine parameters	+	+	+
hemorheology	+	+	+
hemostasis	+	+	+
immunological indicators	+	+	+

CeD: celiac disease; CDAI: Crohn's Disease Activity Index; GFD: gluten-free diet; GIP: gluten-immunogenic peptides; GSRS: Gastrointestinal Symptoms Rating Scale; IBD: inflammatory bowel disease

Detailed history (including medications for preceding three months) and risk factors of venous and arterial thrombotic events will be covered with a 15-minute thrombophilia questionnaire (administered by a person with a medical degree).

The Gastrointestinal Symptoms Rating Scale is a tool designed to assess the severity of gastrointestinal symptoms on a scale of 1 to 7 (administered by a person with a medical degree).[55]

Disease activity in IBD will be estimated with either the (modified) Mayo Score[56] or Crohn's Disease Activity Index[57] in patients with ulcerative colitis and Crohn's disease, respectively, while tissue transglutaminase (tTG) levels will be used to measure the activity of CeD. (Scores will be determined by the gastroenterologist enrolling the patient.)

Dietary adherence of CeD patients will be estimated through (1) a dietary interview conducted by a trained dietitian on a scale of 1 to 10, (2) self-reporting,[58] (3) a test measuring knowledge of gluten-free foods, (4) urine GIP detection (details in the text), and (5) celiac-specific serology (tTG and endomysium antibody levels (EMA)).

All laboratory tests will be performed in the same laboratory (University of Pécs, Hungary) from venous blood. Blood samples will be collected in plastic tubes prospectively (2 x BD Vacutainer 10.0 ml (red), 2 x BD Vacutainer 6.0 ml (purple), 1 x BD Vacutainer 3.0 ml (pink), 1 x BD Vacutainer 2.7 ml (blue), and 1 x BD Seditainer 5.0 ml (black) for a total of 42.7 ml blood from each patient (BD, USA)).

We will measure:

- routine laboratory parameters: bilirubin, urea, creatinine, cholesterol (total, high-density and low-density lipoproteins), triglyceride, aspartate, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total protein, albumin, immunoglobulins, C-reactive protein, vitamin B_{12}, homocysteine, blood counts, and erythrocyte sedimentation

- immunological indicators: antiphospholipid antibodies (lupus anticoagulant, cardiolipin IgG/A/M, B2-glycoprotein-I IgG/A/M, prothrombin IgG/A/M) and celiac-specific antibodies (tTG IgA/G, EMA IgA).
- hemostatic parameters: prothrombin, thrombin time, activated partial thromboplastin time, fibrinogen, antithrombin activity, protein C activity, and protein S activity
- hemorheological parameters: erythrocyte aggregation by Myrenne aggregometer (model MA-1, Myrenne GmbH, Roetgen, Germany) and Laser-assisted Optical Rotational Cell Analyzer (LORCA, R&R Mechatronics, Hoorn, The Netherlands); erythrocyte deformability with laser-diffraction ektacytometry with a LORCA; and viscosity of whole blood and plasma by Brookfield DV-III Ultra LV Programmable rotational viscometer (Brookfield Engineering Labs; Middleboro, Mass., USA).

Strict adherence will be kept during the hemorheological tests to the guidelines proposed by the International Expert Panel for Standardization of Hemorheological Methods.[59] The fact that equipment for hemorheological measurements is not available in other centres in Hungary and that blood samples must be processed within two hours of sampling without freezing restricted our expansion of this project to a multicentre study.

An extra tube will be collected and stored for further hemostatic measurements (e.g., clotting factors) if any abnormality of parameters measured is detected.

Midstream urine (at least 100 ml) will be collected in sterile urine sample containers. Samples will be stored at 4°C until transfer to the Biobank at the Institute for Translational Medicine, University of Pécs Medical School, on the day of sampling, where samples will be deep frozen at -80°C. After preparation, urine GIP detection will be performed with Biomedal (Spain) products.

Outcomes

1. Primary
   - Hemorheological test results (erythrocyte aggregation and deformability, whole blood and plasma viscosity).
2. Secondary
   - Hemostatic test results (antithrombin, protein C, protein S).

Target number of patients

This is a two-phase study. In the first phase, we will enrol 50 CeD and 50 IBD patients with 50 age- and sex-matched control subjects; the case-control ratio will be 1:1:1, respectively. Then, an interim analysis will be performed to calculate the power for the analyses of the outcomes. If the power exceeds 80%, recruitment will be considered completed; otherwise, recruitment will continue until the desired power is reached.

Patient and Public Involvement

Before starting recruitment, randomly selected CeD and IBD patients reviewed the questionnaires and the information sheet designed to share details of the study for participants to facilitate better understanding.

Blinding

Blinding of personnel included in the study is presented in Table 4.

Table 4. Blinding of personnel included in the study

	Physician enrolling patient	Physician administering questionnaires	Dietitian	Laboratory personnel
Disease activity	N/A	Blinded	Blinded	Blinded
Questionnaires	Blinded*	N/A	Blinded	Blinded
Dietary interview	Blinded*	Blinded	N/A#	Blinded
Laboratory measures	Blinded*	Blinded	Blinded	N/A

N/A: not applicable.
*The treating physician will immediately access data for safety reasons and act accordingly. Patients will be informed of the laboratory results in a letter.
#Dietary education will be provided based on dietary adherence.

Data management

A subject identification number will be provided consecutively to every patient after inclusion. Subject identification numbers with sensitive data on patients (including the name, insurance number, and date of enrolment) will be stored in a locked file separately from other data. De-identified data will be added to the source documentation stored in locked cabinets. Source documentation will be entered in an electronic case report file (e-CRF). The Principal Investigators will ensure that the data in an e-CRF are accurate, complete, and legible (range checks for data values). E-CRFs will be stored on a secured server at the Institute for Translational Medicine, University of Pécs Medical School. Access to data will be restricted through a password system to personnel involved in data management. A three-level data

check will be continuously performed, and final data will be finally approved by the Principal Investigator to ensure data quality.

To ensure precise data collection, administrative and medical staff members will be invited to participate in training sessions to familiarize them with the study requirements, standardized data recording, and biological specimen collection.

The de-identified dataset will be delivered for the purpose of sharing on request.

Statistical Analysis

First, descriptive statistics will entail a graphical presentation of data. Continuous variables will be reported as a central tendency with a measure of dispersion, while categorical variables will be reported as absolute and relative frequencies. Then, data will be analyzed with Student's tests, methods of Variance Analysis, and regression models if data are normally distributed; otherwise, non-parametric tests will be introduced. Chi-square or Fisher's tests will be used to analyze categorical variables. Multivariate analysis will be used to explore the association between thrombotic risk factors and primary outcomes. A probability of less than .05 indicates a statistically significant difference between groups.

Only patients with a full dataset in their hemorheological and hemostatic profile will be included in the analysis. The following comparisons will be done: CeD vs. control, tTG+ CeD vs. tTG- CeD, IBD vs. control, active IBD vs. remitting IBD.

An interim analysis is planned after recruiting the target number of the first phase to calculate power. Audits are not necessary due to the case-control design.

Biobank and accessory research

After laboratory analysis, urine and blood (whole blood and plasma, at least 1 ml each) residues will be stored in the Institute for Translational Medicine Biobank at -80°C for future studies (for at least five years). Additional samples will not be taken for storage purposes. Containers will be labelled with the subject identification number, and samples will be completely de-identified.

CeD patients will be offered an opportunity to participate in the "Monitoring the prevalence, symptoms, complications, and family history of celiac disease and the effect of a gluten-free diet – Celiac registry" research project (approved by the Scientific and Research Ethics Committee of the Medical Research Council, Ref No 45098-2/2016/EKU).

Protocol amendments and disseminating policy

This protocol is the first version completed on 30 May 2018. If required, the online version will be updated in the ISRCTN registry. Major modifications should be permitted by the Regional and Local Research Ethics Committee.

The trial status is ongoing; recruitment began on 1 May 2018. The expected date of completion is 31 May 2019.

ETHICS AND DISSEMINATION

The study was approved by the Regional and Local Research Ethics Committee, University of Pécs (Ref No 6917). Publication in a high-impact peer-reviewed journal is planned. We will adhere to authorship criteria for manuscripts submitted for publication set by the International Committee of Medical Journal Editors.

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AUTHORS' CONTRIBUTIONS

JB is the Principal Investigator. ZS is the Trial Coordinator. ZS, PH, JB, ÁV, and KT conceptualized the study, drafted, and revised this manuscript. NF and EB planned and drafted the statistical analysis. PS, JB, and ÁV provided us with special expertise in the management of celiac disease and inflammatory bowel patients. BC and PK are performing the hemorheological measurements and interpreting the results. AH, ÁN, TB, and MTF provided us with special expertise in hemostatic and immunological measurements. BK is contributing significantly to the biochemical analyses. IV planned and is carrying out the dietary assessment of the celiac patients. KM, AS, ZS, and PH are responsible for data management, administrative coordination, and biological sampling; they drafted and revised the manuscript. All the authors have read and approved the final manuscript.

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COMPETING INTEREST STATEMENT

Nothing to declare.

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Hemorheological and hemostatic alterations in celiac disease and inflammatory bowel disease in comparison with non-celiac, non-IBD subjects (HERMES): A case-control study protocol

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Hemorheological and hemostatic alterations in celiac disease and inflammatory bowel disease in comparison with non-celiac, non-IBD subjects (HERMES): A case-control study protocol

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Abstract

Introduction

Hemorheological and hemostatic changes predispose to the development of arterial and venous thrombotic events; however, limited information is available on the status of these changes in celiac disease (CeD) and inflammatory bowel disease (IBD). In this study, we aim to describe the hemorheological and hemostatic profiles of CeD and IBD patients in a Hungarian cohort of patients to investigate whether any alterations contribute to elevated thrombotic risk.

Methods and analysis

This is a case-control study involving newly diagnosed and followed CeD and IBD patients with age- and sex-matched non-CeD, non-IBD subjects with an allocation ratio of 1:1:1.

After informed consent is obtained, a detailed medical history will be collected, including venous and arterial thrombotic risk factors and medications. Symptoms in CeD patients will be assessed with the Gastrointestinal Symptoms Rating Scale, and disease activity in IBD patients will be determined by disease-specific scores. Dietary adherence will be assessed among CeD patients with a thorough interview together with a measurement of self-reported adherence, dietary knowledge, and urine analysis (detection of gluten immunogenic peptides). In addition to routine laboratory parameters, hemorheological (i.e., erythrocyte deformability and aggregation, viscosity of whole blood and plasma) and hemostatic parameters (e.g., protein C, protein S, and antithrombin) with immunological indicators (i.e., celiac-specific serology and antiphospholipid antibodies) will be measured from venous blood for every participant.

Primary and secondary outcomes will be hemorheological and hemostatic parameters, respectively. Univariate and multivariate statistics will be used to compare CeD and IBD patients to control subjects. Subgroup analysis will be performed by disease type in IBD, (Crohn's disease and ulcerose colitis), dietary adherence in CeD, and disease activity in IBD and CeD.

Ethics and dissemination

The study was approved by the Regional and Local Research Ethics Committee, University of Pécs (Ref No 6917). Findings will be disseminated at research conferences and in peer-reviewed journals.

Trial registration

ISRCTN49677481.

Key words: celiac disease, inflammatory bowel disease, hemorheology, thrombosis

Strengths and limitations of this study

- Immune-mediated bowel diseases are associated with an increased risk of arterial and venous thrombosis, but specific hemorheological and hemostatic alterations are understudied in celiac disease and incomplete in inflammatory bowel disease.
- This case-control study prospectively recruits newly diagnosed and followed-up cases of celiac disease and inflammatory bowel disease with age- and sex-matched controls (the allocation ratio will be 1:1:1, respectively) to investigate clinical and laboratory alterations predisposing to thrombosis.
- Laboratory tests include the measurement of hemorheological (i.e., erythrocyte aggregation and deformability, plasma and whole blood viscosity), hemostatic parameters (e.g., levels of fibrinogen, prothrombin time, protein C, protein S, and antithrombin), and immunological indicators (e.g., celiac-specific serology and antiphospholipid antibodies).
- Patients will be divided by disease activity into active and inactive.
- Results should be interpreted with caution due to the single-centre nature and case-control design of the study.

INTRODUCTION

Immune-mediated disorders may affect 5–7% of the population.[1] These disorders frequently share pathways in pathogenesis as well as organ manifestations. Celiac disease (CeD) and inflammatory bowel disease (IBD) are systemic disorders, primarily affecting the intestines.[2] They impose a significant burden of complications and concomitant diseases on patients during the disease course.

CeD is a chronic, immune-mediated disorder, which develops upon gluten ingestion in genetically susceptible individuals.[3] Global prevalence of CeD is around 1% with geographical differences ranging from 0.14% up to 5.7%.[3] The clinical presentation can be divided into classic, non-classic, and asymptomatic forms.[4] Diagnosing asymptomatic and atypical cases is challenging but important, because the disease course of these cases may be alike.[5]

IBD – clinically classified as Crohn's disease or ulcerative colitis – is a chronic, relapsing disorder, which develops as a result of the interaction between environmental and genetic

factors, leading to immunological responses and inflammation in the gastrointestinal tract.[6] IBD is a less frequent entity than CeD: the increasing prevalence of ulcerative colitis and Crohn's disease may reach 0.5% and 0.3% in Europe, respectively.[7, 8]

Immune-mediated disorders may be associated with hemorheological[9-11] and hemostatic changes[12-14], thereby contributing to an increased risk of thrombotic events.[15] This increased risk is manifested in CeD[16] and IBD.[17] Mechanisms of thrombophilia in immune-mediated disorders are complex, and acquired factors seem important.[18] An altered hemorheological profile as well as the altered levels or function of pro- and anticoagulant proteins, altered activity of clotting factors contribute to the development of arterial and venous thrombotic events.[19-23]

Clinical presentation of CeD-associated hypercoagulability includes a wide variety of thrombosis at venous sites, pulmonary embolism, atheroembolism (stroke), and obstetric complications.[24, 25] A single retrospective publication examined hemostatic alterations in a small cohort of patients: sporadic cases of protein C and protein S deficiency (due to vitamin K malabsorption), hyperhomocysteinemia, and antiphospholipid antibodies were identified.[26] No studies have assessed the hemorheological changes in CeD. The multifactorial etiology of thrombosis may embrace the interplay of malabsorption (vitamin and mineral deficiencies, e.g., vitamin B_{12} and K deficiency), thrombophilic autoantibodies (anti-tissue transglutaminase (tTG) and antiphospholipid antibodies), hyperhomocysteinemia, endothelial dysfunction, accelerated atherosclerosis, thrombocytosis and thrombocyte dysfunction, hyperviscosity, and genetics.[24, 26-31] Immune-mediated comorbidities ('autoimmune traits'), such as antiphospholipid syndrome, may contribute to the elevated thrombotic risk as well.[29] In addition, ingestion of trace amounts of gluten may maintain a continuous pro-inflammatory response.[32]

IBD is associated with venous thrombosis and pulmonary embolism as well as with the cardiovascular consequences of atherosclerosis, i.e., stroke and myocardial infarction.[33, 34] A significant decline in anticoagulant mechanism is well-established and there are sporadic reports on activity-dependent prothrombotic hemorheological changes.[35-38] However, while individual studies have focused on single outcomes of laboratory parameters, none of them have assessed the complete hemorheological profile of patients.[39-42] Other risk factors include immobilization, surgical interventions, glucocorticoid therapy, vitamin deficiencies, hyperhomocysteinemia, and chronic inflammation alone or in conjunction with the factors above.[43, 44] In the case of IBD, disease activity may be a crucial determinant of thrombotic risk.[34]

Scope and objectives

No studies have assessed hemorheological and hemostatic parameters within a study to provide an overall view of thrombotic risk. Since our knowledge of hemorheological and hemostatic changes is limited in CeD and IBD, this study aims to carry out a comprehensive evaluation of venous and arterial prothrombotic alterations in these pro-inflammatory diseases in a Hungarian cohort of patients.

1. Primary objective
   - to identify a link between prothrombotic hemorheological and hemostatic alterations and two common immune-mediated diseases (CeD and IBD)
2. Secondary objective
   - to investigate the effect of disease activity on the hemorheological and hemostatic profiles of CeD and IBD patients
   - to find an association between the dietary adherence of CeD patients and the hemorheological and hemostatic alterations
   - to assess the modifying effect of immunosuppressant drugs in IBD on the hemorheological and hemostatic profiles

METHODS AND ANALYSIS

Design

This is a case-control study with prospective recruitment of CeD and IBD patients with non-CeD, non-IBD control subjects. The study does not change the routine management of subjects included (for the World Health Organization checklist, see Table 1). The study protocol was planned in accordance with the SPIRIT 2013 Statement.[45]

Table 1. World Health Organization checklist

Data category	Information
Primary registry and trial identifying number	ISRCTN49677481
Date of registration in primary registry	05/03/2018
Secondary identifying numbers	None
Source(s) of monetary or material support	University of Pécs Medical School; Momentum Grant from the Hungarian Academy of Sciences (LP2014-10/2014); Highly Cited Publication Grant (KH 125678) from the National Research Development and Innovation Office; GINOP 2.3.2-15-2016-00048 Stay Alive, EFOP 3.6.2-16-2017-00006 Live Longer, and EFOP-

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Primary sponsor	None
Secondary sponsor(s)	None
Contact for public queries	Zsolt Szakács, MD, szakacs.zsolt@pte.hu
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Public title	Investigation of hemorheological and hemostatic alterations in celiac disease and inflammatory bowel disease in comparison with healthy subjects: A case-control study (HERMES)
Scientific title	Hemorheological and hemostatic alterations in celiac disease and inflammatory bowel disease in comparison with non-celiac, non-IBD subjects: A case-control study (HERMES)
Countries of recruitment	Hungary
Health condition(s) or problem(s) studied	Celiac disease and inflammatory bowel disease
Intervention(s)	Questionnaires (thrombophilia, dietary adherence, disease activity), urine collection (dietary adherence - urine-gluten immunogenic peptide detection), blood collection (hemorheological, hemostatic, and immunological tests complemented with routine laboratory panel)
Key inclusion and exclusion criteria	Inclusion criteria: adult patients (≥18 years of age) suffering from biopsy-confirmed newly diagnosed or treated celiac disease (by ESPHGAN, ACG, WGO guidelines), or from inflammatory bowel disease (by ECCO guidelines), and non-celiac, non-IBD subjects
	Exclusion criteria: chronic diseases (chronic kidney diseases, liver cirrhosis, heart failure, active malignant diseases), acute diseases within 2 weeks of inclusion, pregnancy, thrombotic events within 1 year, systematic lupus erythematosus, and use of oral anticoagulants or antiplatelet therapy
Study type	Observational
Date of first enrolment	30/5/2018
Target sample size	First phase: 50 celiac and 50 IBD patients plus control (1–3 for each patient). Second phase: target number is determined by power calculation.
Recruitment status	Ongoing
Primary outcome(s)	Hemorheological test results
Key secondary outcomes	Hemostatic test results

Trial organization and steering committee

The Centre for Translational Medicine at the University of Pécs, which was established to advance medical research in gastroenterology, is the coordinator and designer of the HERMES study. The centre is experienced in running investigator-initiated clinical trials.[46] A steering committee will be set up to supervise the entire study process. The Principal Investigator (JB) and the Trial Coordinator (ZS) are responsible for organizing patient recruitment, data

collection, sample collection, shipping, and storage, biochemical analysis, and the publication of study results.

Population and eligibility

We will include CeD patients, IBD patients, and non-CeD, non-IBD control subjects. Eligibility criteria will be as follows:

a. Inclusion criteria (applies to all subjects)

- Blood collection must be indicated with medical conditions
- Signed informed consent

b. Inclusion criteria (applies to specific cohorts of patients)

- CeD patients: biopsy-confirmed newly diagnosed or followed patients (with or without adhering to a gluten-free diet) aged ≥18 years; the establishment of a diagnosis should meet the current guidelines (ESPHGAN, ACG).[3, 47, 48]
- IBD patients: newly diagnosed or followed-up patients (with active or remitting disease) aged ≥18 years (not following a gluten-free diet); the establishment of a diagnosis should meet the current guidelines (ECCO).[49, 50]
- Non-CeD, non-IBD control subjects: individuals aged ≥18 years (not following a gluten-free diet) in whom CeD and IBD can be excluded according to the recent guidelines. [3, 47-50]

c. Exclusion criteria (applies to all subjects)

- Chronic conditions:
  - Estimated glomerular filtration rate calculated with CKD-EPI formula is <60ml/min/1.73m^2 (CKD3 or more severe kidney failure)
  - Liver cirrhosis in Child–Pugh B–C
  - Heart failure (NYHA III–IV)
  - Active malignant diseases
- Any acute diseases or invasive procedures within two weeks of recruitment (e.g., systemic infection, surgery, or major trauma)
- Thrombotic events within 1 year of recruitment
- Ongoing oral anticoagulant therapy (vitamin K antagonists) and/or antiplatelet drugs
- Confirmed systemic lupus erythematosus
- Pregnancy
- Patients unable to understand the essentials of the informed consent

Flow and timing

All subjects at our academic hospital for a planned check-up or referred to the center for diagnostic purposes will be recruited consecutively. The place of recruitment will be the Division of Gastroenterology, First Department of Medicine, University of Pécs Medical School. This tertiary centre provides professional gastroenterological care for about 300,000 inhabitants in Baranya County, Hungary.

Recruitment of the study population will be managed in two phases (see 'Target number of patient' section), with the expected recruiting period being between May 2018 and May 2019 (covering one year). Table 2 shows the timeline of the study. Patients will be provided with an information sheet and must provide written consent before sampling. Informed consent will be obtained by personnel with a medical degree. Participants may withdraw from the study for any reason at any time. Consent forms and other related documents will be accessible at https://tm-centre.org.

Table 2. Schedule for the study

TIME POINT	Study period				
	Enrolment	Allocation	Post-allocation		
	-1 hour	0	+1 hour	+1.5 hour	+2 hour
ENROLMENT:					
Eligibility screen	x				
Informed consent	x				
Allocation		x			
INTERVENTION:					
Interview and questionnaire			x		
Urine collection				x	
Blood collection				x	
ASSESSMENT					
Symptom scores and disease activity			x		
Thrombophilia questionnaire			x		
Dietary adherence			x		
Blood analysis[#]					x
Urine analysis[#]					=>[*]

[*]samples will be deep frozen until all participants have been recruited
[#]after analysis, blood and urine residues will be stored in the biobank

Patients will be monitored by our professional data management team throughout the entire process of data and biological sample collection to ensure perfect adherence to protocol. Written feedback will be provided to patients on the results of the laboratory tests and dietary evaluation. If findings indicate, patients will be referred to their general practitioners or a specialist for further investigation and management.

Measurements

All samples will be collected and questionnaires will be administered within two hours after allocation. Actions for each group are defined and listed in Table 3.

Table 3. Actions within study

	CeD patients	IBD patients	Control subjects
Thrombophilia questionnaire	+	+	+
GSRS	+	-	+
Dietary interview and GFD adherence tests	+	-	+
Mayo Score/CDAI	-	+	+
Urine GIP detection	+	-	+
Laboratory measures			
routine parameters	+	+	+
hemorheology	+	+	+
hemostasis	+	+	+
immunological indicators	+	+	+

CeD: celiac disease; CDAI: Crohn's Disease Activity Index; GFD: gluten-free diet; GIP: gluten-immunogenic peptides; GSRS: Gastrointestinal Symptoms Rating Scale; IBD: inflammatory bowel disease

Detailed history (including medications for preceding three months) and risk factors of venous and arterial thrombotic events will be covered with a 15-minute thrombophilia questionnaire (administered by a person with a medical degree).

The Gastrointestinal Symptoms Rating Scale is a tool designed to assess the severity of gastrointestinal symptoms on a scale of 1 to 7 (administered by a person with a medical degree).[51]

Disease activity in IBD will be estimated with either the (modified) Mayo Score[52] or Crohn's Disease Activity Index[53] in patients with ulcerative colitis and Crohn's disease, respectively, while tissue transglutaminase (tTG) levels will be used to measure the activity of CeD. (Scores will be determined by the gastroenterologist enrolling the patient.)

Dietary adherence of CeD patients will be estimated through (1) a dietary interview conducted by a trained dietitian on a scale of 1 to 10, (2) self-reporting,[54] (3) a test measuring knowledge of gluten-free foods, (4) urine GIP detection (details in the text), and (5) celiac-specific serology (tTG and endomysium antibody levels (EMA)).[55] Patients will be divided into those with good and poor dietary adherence based on the complex assessment of the above-mentioned data.

All laboratory tests will be performed in the same laboratory (University of Pécs, Hungary) from venous blood. Blood samples will be collected in plastic tubes prospectively (2 x BD Vacutainer 10.0 ml (red), 2 x BD Vacutainer 6.0 ml (purple), 1 x BD Vacutainer 3.0 ml (pink), 1 x BD Vacutainer 2.7 ml (blue), and 1 x BD Seditainer 5.0 ml (black) for a total of 42.7 ml blood from each patient (BD, USA)).

We will measure:

- routine laboratory parameters: bilirubin, urea, creatinine, cholesterol (total, high-density and low-density lipoproteins), triglyceride, aspartate, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total protein, albumin, immunoglobulins, C-reactive protein, vitamin B_{12}, folic acid, homocysteine, blood counts, and erythrocyte sedimentation
- immunological indicators: antiphospholipid antibodies (lupus anticoagulant, cardiolipin IgG/A/M, B2-glycoprotein-I IgG/A/M, prothrombin IgG/A/M) and celiac-specific antibodies (tTG IgA/G, EMA IgA).
- hemostatic parameters: prothrombin, thrombin time, activated partial thromboplastin time, fibrinogen, antithrombin activity, protein C activity, and protein S activity
- hemorheological parameters: erythrocyte aggregation by Myrenne aggregometer (model MA-1, Myrenne GmbH, Roetgen, Germany) and Laser-assisted Optical Rotational Cell Analyzer (LORCA, R&R Mechatronics, Hoorn, The Netherlands); erythrocyte deformability with laser-diffraction ektacytometry with a LORCA; and viscosity of whole blood and plasma by Brookfield DV-III Ultra LV Programmable rotational viscometer (Brookfield Engineering Labs; Middleboro, Mass., USA). The Case Report Form providing data about the measurements is presented in Supplementary material.

Strict adherence will be kept during the hemorheological tests to the guidelines proposed by the International Expert Panel for Standardization of Hemorheological Methods.[56] The fact that equipment for hemorheological measurements is not available in other centres in Hungary

and that blood samples must be processed within two hours of sampling without freezing restricted our expansion of this project to a multicentre study.

An extra tube will be collected and stored for further hemostatic measurements (e.g., clotting factors) if any abnormality of parameters measured is detected.

Midstream urine (at least 100 ml) will be collected in sterile urine sample containers. Samples will be stored at 4°C until transfer to the Biobank at the Institute for Translational Medicine, University of Pécs Medical School, on the day of sampling, where samples will be deep frozen at -80°C. After preparation, urine GIP detection will be performed with Biomedal (Spain) products.

Outcomes

1. Primary
    - Hemorheological test results (erythrocyte aggregation and deformability, whole blood and plasma viscosity).
2. Secondary
    - Hemostatic test results (antithrombin, protein C, protein S), folic acid, and homocysteine levels.

Target number of patients

This is a two-phase study. In the first phase, we will enrol 50 CeD and 50 IBD patients with 50 age- and sex-matched control subjects; the case-control ratio will be 1:1:1, respectively. Then, an interim analysis will be performed to calculate the power for the analyses of the outcomes. If the power exceeds 80%, recruitment will be considered completed; otherwise, recruitment will continue until the desired power is reached.

Patient and Public Involvement

Before starting recruitment, randomly selected CeD and IBD patients reviewed the questionnaires and the information sheet designed to share details of the study for participants to facilitate better understanding.

Blinding

Blinding of personnel included in the study is presented in Table 4.

Table 4. Blinding of personnel included in the study

	Physician enrolling patient	Physician administering questionnaires	Dietitian	Laboratory personnel
Disease activity	N/A	Blinded	Blinded	Blinded
Questionnaires	Blinded*	N/A	Blinded	Blinded
Dietary interview	Blinded*	Blinded	N/A#	Blinded
Laboratory measures	Blinded*	Blinded	Blinded	N/A

N/A: not applicable.
*The treating physician will immediately access data for safety reasons and act accordingly. Patients will be informed of the laboratory results in a letter.
#Dietary education will be provided based on dietary adherence.

Data management

A subject identification number will be provided consecutively to every patient after inclusion. Subject identification numbers with sensitive data on patients (including the name, insurance number, and date of enrolment) will be stored in a locked file separately from other data. De-identified data will be added to the source documentation stored in locked cabinets. Source documentation will be entered in an electronic case report file (e-CRF). The Principal Investigators will ensure that the data in an e-CRF are accurate, complete, and legible (range checks for data values). E-CRFs will be stored on a secured server at the Institute for Translational Medicine, University of Pécs Medical School. Access to data will be restricted through a password system to personnel involved in data management. A three-level data check will be continuously performed, and final data will be finally approved by the Principal Investigator to ensure data quality.

To ensure precise data collection, administrative and medical staff members will be invited to participate in training sessions to familiarize them with the study requirements, standardized data recording, and biological specimen collection.

The de-identified dataset will be delivered for the purpose of sharing on request.

Statistical Analysis

First, descriptive statistics will entail a graphical presentation of data. Continuous variables will be reported as a central tendency with a measure of dispersion, while categorical variables will be reported as absolute and relative frequencies. Then, data will be analyzed with Student's tests, methods of Variance Analysis, and regression models if data are normally distributed; otherwise, non-parametric tests will be introduced. Chi-square or Fisher's tests will be used to analyze categorical variables. Multivariate analysis will be used to take the most important thrombotic factors into account (e.g., the use of oral contraceptives and immunosuppressants,

previous thrombotic history, smoking, comorbidities). A probability of less than .05 indicates a statistically significant difference between groups.

Only patients with a full dataset in their hemorheological and hemostatic profile will be included in the analysis. The following comparisons will be done: CeD vs. control, tTG+ CeD vs. tTG- CeD, CeD with good dietary adherence vs. CeD with poor dietary adherence, IBD vs. control, active IBD vs. remitting IBD, and Crohn's disease vs. ulcerative colitis.

An interim analysis is planned after recruiting the target number of the first phase to calculate power. Audits are not necessary due to the case-control design.

Biobank and accessory research

After laboratory analysis, urine and blood (whole blood and plasma, at least 1 ml each) residues will be stored in the Institute for Translational Medicine Biobank at -80°C for future studies (for at least five years). Additional samples will not be taken for storage purposes. Containers will be labelled with the subject identification number, and samples will be completely de-identified.

CeD patients will be offered an opportunity to participate in the "Monitoring the prevalence, symptoms, complications, and family history of celiac disease and the effect of a gluten-free diet – Celiac registry" research project (approved by the Scientific and Research Ethics Committee of the Medical Research Council, Ref No 45098-2/2016/EKU).

Protocol amendments and disseminating policy

This protocol is the first version completed on 30 May 2018. If required, the online version will be updated in the ISRCTN registry. Major modifications should be permitted by the Regional and Local Research Ethics Committee.

The trial status is ongoing; recruitment began on 1 May 2018. The expected date of completion is 31 May 2019.

DISCUSSION

Recent guidelines on CeD do not make any recommendations on how to prevent and manage thrombotic events in CeD patients.[3, 47] Gluten-free diet, which is the only approved treatment of the disease, may reduce or eliminate some thrombotic risk factors (e.g., consequences of malabsorption and chronic inflammation) but it is uncertain whether the thrombotic risk completely normalizes.[57] With respect to malabsorption, intestinal mucosa does not recover in a high fraction of patients despite a long-term strict diet, particularly in those diagnosed in

the adulthood.[58] Whether CeD patients after a thrombotic event would benefit from a lifelong anticoagulation therapy has remained unclear. The need for thromboprophylaxis under prothrombotic circumstances, such as hospitalization, pregnancy, or immobilization should be further investigated.

IBD guidelines recommend that thromboprophylaxis should be considered in all in- and outpatients with an active disease.[59, 60] In addition to disease severity, the choice of treatment influences the thrombotic risk as well.[17] A tool of personalized thrombotic risk stratification including objective laboratory markers is awaited.

Our results can contribute to expanding our knowledge on the prothrombotic pathophysiological alteration in CeD and IBD, thereby providing the basis for future research.

ETHICS AND DISSEMINATION

The study was approved by the Regional and Local Research Ethics Committee, University of Pécs (Ref No 6917). Publication in a high-impact peer-reviewed journal is planned. We will adhere to authorship criteria for manuscripts submitted for publication set by the International Committee of Medical Journal Editors.

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AUTHORS' CONTRIBUTIONS

JB is the Principal Investigator. ZS is the Trial Coordinator. ZS, PH, JB, ÁV, and KT conceptualized the study, drafted, and revised this manuscript. NF and EB planned and drafted the statistical analysis. PS, JB, BE, and ÁV provided us with special expertise in the management of celiac disease and inflammatory bowel patients. BC and PK are performing the hemorheological measurements and interpreting the results. AH, ÁN, TB, and MTF provided us with special expertise in hemostatic and immunological measurements. BK is contributing significantly to the biochemical analyses. IV planned and is carrying out the dietary assessment of the celiac patients. KM, AS, ZS, and PH are responsible for data management, administrative coordination, and biological sampling; they drafted and revised the manuscript. All the authors have read and approved the final manuscript.

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COMPETING INTEREST STATEMENT

Nothing to declare.

Case Report Form - hemorheological studies

HERMES

Date and time (MM:HH DD:MM:YYYY): ……..

Hematocrit (%): .....

Red blood cell aggregation (Myrenne aggregometer)[1]:

	First sample			Sacond sample			Mean
M index							
M1 index							

Red blood cell aggregation (LORCA)[2]:

AI (%): ..... t ½ (s): ..... ɣ: (1/s) .....

Red blood cell deformability (LORCA)[3]:

EI_{30}	$EI_{16.87}$	$EI_{9.49}$	$EI_{5.33}$	EI_{3}	$EI_{1.69}$	$EI_{0.95}$	$EI_{0.53}$	$EI_{0.3}$

Viscosity of whole blood (Brookfield viscometer, mPa·s)[4]: ……

Viscosity of plasma (Brookfield viscometer, mPa·s)[4]: ……

[1]The Myrenne aggregometer (model MA-1, Myrenne GmbH, Roetgen, Germany) consists of a laser diode, a transparent upper plate, and a transparent lower rotating cone (i.e., shearing unit). A sample (30 µl red blood cell suspension) is placed between the gap of the upper plate and the rotating cone. Then, the rotating cone shears the red blood cells at 600 s^{-1} to disaggregate pre-existing aggregates. The cone then suddenly stops moving (**M index or aggregation at stasis**) or continues to rotate (**M1 index or aggregation at low shear**) and shear at 3 s^{-1} while measuring the intensity of transmitted infrared light for 10 s. If the red blood cells tend to aggregate, the light transmittance of the sample increases (as well as M and M1 indices). Measurements are carried out at room temperature three times from two samples.

[2]The Laser-assisted Optical Rotational Cell Analyzer (LORCA, R&R Mechatronics, Hoorn, The Netherlands) consists of a glass cup and a perfectly fitting bob with a 0.3 mm gap in between. The red blood cell suspension is placed in this gap. The disaggregation of the sample is carried out at 500 s^{-1} shear. Following the release, a red laser beam is directed to the sample and the reflection is measured by photodiodes for 120 s. **Aggregation index (AI)** is calculated by the integral of the change in in reflection (intensity of light) during the first 10 s corrected to the possible maximal change. **T½** defines the time required for achieving the half of the maximal aggregation. The threshold shear rate is the lowest shear rate that can maintain complete disaggregation (**$\dot{\gamma}$**). Measurements are carried out at room temperature.

[3]Red blood cells are exposed to shear stress on ektacytometry (with LORCA, R&R Mechatronics, Hoorn, The Netherlands), then the change in shape is quantified. LORCA consists of a rotating glass cup with a fitting bob, which provides a nearly homogenous shearing field. Low hematocrit (0.2%) is required for the measurement; therefore, we suspend them in a medium with known viscosity (25 µl blood/5 ml volume). The exposure of red blood cells to shear stress results in the transition of the normally biconcave shape to an ellipsoid one. The deformed cells are examined with a laser beam causing diffraction. The **elongation index (EI)** quantifies the deformation of the red blood cells (EI=(A-B)/(A+B), where A and B represent the major and the minor axes of the ellipsoids). EI can be determined at various shear stresses, the set of shear stress-elongation index is the so-called ektacytogram. Shear stresses range from 0.3 to 30.0 kPa (e.g., **$EI_{0.3}$, EI_3**). Measurements are carried out at room temperature.

[4]Viscosity of the whole blood and plasma is determined by Brookfield DV-III Ultra LV Programmable rotational viscometer (Brookfield Engineering Labs; Middleboro, Mass., USA). A spindle is immersed in the sample that the torque required to rotate it is measured. The **torque** is proportional to the viscosity measured at mid-shear (90 s^{-1} or 12 rpm) in our study. Measurements are carried out at 37°C .