Haemorheological, Natural Anticoagulant and Homocysteine Profiles in Coeliac Disease: A Case-control Study

PhD Thesis

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3. Synoptics

Coeliac disease (CeD) is an immune-mediated disease, which develops upon gluten ingestion in genetically predisposed individuals. CeD patients are at a higher risk of acute arterial and venous thrombotic events. In this study, we aimed to assess if prothrombotic alterations characterize CeD patients, with special focus on rheological properties of blood and its cellular elements, i.e. haemorheology.

This study is a case-control study registered under registration number ISRCTN49677481. Cases were adult, biopsy-verified CeD patients, controls were subjects in whom CeD was excluded. All participants were free from acute and advanced chronic diseases. In addition to a routine set of laboratory studies, we measured haemorheological parameters, the activity of natural anticoagulants (protein C, protein S and antithrombin) and the level of homocysteine. The haemorheological profile included haematocrit, whole blood viscosity (WBV), plasma viscosity, fibrinogen, erythrocyte aggregation (EA) and erythrocyte deformability (ED, described with the elongation index at high and low shear stresses). We compared CeD patients to control subjects with uni- and multivariate statistics and investigated the effects of dietary adherence in CeD.

After matching by age and sex, we included 50 CeD patients—47 of which were on a gluten-free diet ≥1 year—and 50 control subjects in analysis. Elongation index at high shear stresses was significantly lower in CeD patients compared to control subjects, and CeD proved to be an independent predictor of the index; suggesting an impaired ED in CeD. Besides, CeD patients had a significantly higher level of fasting homocysteine compared to control subjects (median 9.0 vs 8.7 μmol/L, respectively; $p=0.040$). The impairment in ED at high shear stresses seemed to be only partly dependent on dietary adherence. EA and WBV were consistently shifted towards a prothrombotic direction in CeD patients with poor dietary adherence compared to those with good dietary adherence; highlighting the importance of a strict gluten-free diet regarding EA and WBV. The other outcome parameters were similar between the groups and were independent of dietary adherence.

In summary, ED at high shear stresses is impaired (mainly independently of dietary adherence) and the level of homocysteine is higher (irrespective of dietary adherence) in CeD compared to the control group. In contrast, poor dietary adherence results in impaired EA and higher WBV in CeD. These diet-dependent and -independent prothrombotic changes can contribute to the elevated CV risk in CeD, and raise the need for the introduction of preventive measures.

2. Introduction

Coeliac disease (CeD) is a systemic immune-mediated disease, which develops upon gluten ingestion in genetically predisposed individuals. The pooled global prevalence of biopsy-confirmed CeD is 0.7% (95% confidence interval [CI]: 0.5–0.9%), whereas the pooled global seroprevalence of CeD is 1.4% (CI: 1.1–1.7%).

2.1 Pathomechanism of coeliac disease

Environmental triggers (alimentary gluten) and genetic predispositions (human leukocyte antigen [HLA]) are both essential in the pathogenesis of CeD.

Wheat gluten, mixture of storage proteins, can be divided into fractions by solubility: prolamines (α, β, γ, and ω gliadin) are ethanol-soluble whereas glutenins are water-soluble. Other grains synthesize other types of storage proteins (referred to as gluten as well in the everyday language). Wheat gliadin and the storage proteins of other plants have proline- and glutamine-rich sequences, resilient to cleavage by the repertoire of human enzymes, so that their complete degradation to amino acids is impossible in the gut. A broad set of these peptides—the so-called gluten immunogenic peptides (GIP)—contains epitopes capable for triggering an immune response in genetically susceptible individuals.

Concordance of CeD reaches 75–86% among monozygotic twins, whereas first-degree relatives are at 10–15% risk of developing CeD. The role of HLA class II heterodimers in the pathomechanism is extensively studied. CeD patients carry HLA-DQ2 in 90–95% and -DQ8 in 5–10%.

After degradation of gluten by human digestive enzymes and the gut flora to GIPs, the peptides cross the small intestinal epithelium and enter the lamina propria. GIPs gain negative charges through deamidation by tissue transglutaminase enzyme type 2, so that their affinity to bind HLA increases. Deamidated GIPs are presented on HLA-DQ2 or -DQ8 heterodimers by the activated dendritic cells to CD4+ T-cells, starting to produce a set of cytokines, which activate intraepithelial lymphocytes, causing local mucosal damage ending up in total villous atrophy. Besides, plasma cells start forming antibodies—most importantly, anti-tissue transglutaminase antibody (TGA) and anti-endomysial antibody (EMA)—and release them into the circulation, triggering consequences throughout the entire human body.

4.2 Clinical characteristics and diagnosis of coeliac disease

The diagnosis of CeD relies on four pillars: (1) signs and symptoms, (2) serology, (3) histology and (4) genetic testing.

(ad 1) We differentiate classical (with malabsorption), non-classical (without malabsorption) and asymptomatic CeD based on the 2012 Oslo consensus conference. (ad 2) The introduction of CeD-specific serology was a breakthrough in diagnostics as it, unlike the intestinal biopsy, is suitable for quick and non-invasive mass screening. Today, the measurement of TGA and EMA are the gold standards. (ad 3) Villous atrophy is considered a pathognomonic histological feature of CeD. In 1992, Marsh had proposed a simple histopathological classification: Marsh 0–normal, Marsh 1–intraepithelial lymphocytosis, Marsh 2–crypt hyperplasia and Marsh 3–villous atrophy. (ad 4) HLA-DQ2 or -DQ8 is present in nearly all CeD patients. Of note, HLA-typing can be used primarily for ruling-out purposes because many people are positive for these haplotypes in the general population, but less than one-tenth of them develop CeD.

Evidence-based guidelines are available for diagnosing CeD in childhood and adulthood. In adults, CeD is suspected based on clinical clues. Duodenal histological sampling is mandatory to confirm the diagnosis. In children, duodenal biopsy can be omitted if certain conditions are satisfied.

2.3 Treatment of coeliac disease: the gluten-free diet

Although having an effective pharmacological treatment is an appealing possibility; today, the only evidence-based treatment option is the complete exclusion of alimentary gluten. The initiation of a lifelong gluten-free diet (GFD) is recommended immediately after the confirmation of the diagnosis of CeD. The complete exclusion of dietary gluten results in a vast improvement in gastrointestinal symptoms in the majority of CeD patients.

As part of a GFD, products containing wheat, barley and rye should be avoided, whereas, proven by a meta-analysis of randomized controlled trials, the intake of oats can be considered safe for the majority of CeD patients. Even 50–100 mg daily intake of gluten can be harmful (for reference, this is the amount of gluten in about a dozen of crumbs of bread).

Regular assessment of dietary adherence and dietary education are of utmost importance in CeD. The current gold standards for evaluation of dietary adherence include histology, serology and dietary review through interview. A promising ancillary instrument is the measurement of GIPs, which can be detected from faecal or from urine samples.

Although a GFD is the only effective therapeutic option, the nutrient profile of gluten-free products falls far from the optimal. GFD is a low-fibre, high-carb and high-fat diet compared to the average gluten-containing diet. The easy access to gluten-free foods and the improving absorption readily lead to weight gain, which is desirable in the underweight but

undesirable with a normal body mass. If we take into account patients with the increasingly common non-classical phenotype, who usually have normal body weight, obesity and metabolic syndrome developing during a GFD is—and will continue to be—a serious and common problem in the twenty-first century.

2.4 Mortality and cardiovascular diseases in coeliac disease

The overall mortality is increased in CeD. The excess mortality mainly results from lymphoproliferative malignancies and cardiovascular (CV) diseases. To date, in CeD, nine studies reported on CV mortality, of which those recruiting a vast number of subjects detected a significant increase in CV mortality with CeD (Table 1). Risk of stroke, cerebrovascular mortality and major adverse cardiac events were reported to occur more frequent in CeD. The risk of venous thromboembolism was inconsistently elevated across studies.

Table 1. Adjusted cardiovascular mortality in coeliac disease

Studies in chronological order	Country	Age group	N^0 of CeD patients	Relative measure (95% CI)
Corrao et al. 2001	Italy	Adults	1,072	SMR: 0.7 (0.3–1.5)
Peters et al. 2003	Sweden	Mixed	10,032	**SMR: 1.6 (1.4–1.8)**
Viljamaa et al. 2006	Finland	Mixed	781	SMR: 1.2 (0.83–1.68)
Ludvigsson et al. 2009	Sweden	Mixed	29,096	**HR: 1.19 (1.11–1.28)**
Grainge et al. 2011	UK	Mixed	1,092	SMR: 1.12 (0.82–1.50)
Abdul Sultan et al. 2015	UK	Mixed	10,825	**CIF: –1.4% (–1.9 to –0.9%)**
Holmes and Muirhead 2018	UK	Adults	2,174	SMR: 1.23 (0.98–1.51)
Lebwohl et al. 2020	Sweden	Mixed	49,829	**HR: 1.08 (1.02–1.13)**
Koskinen et al. 2020	Finland	Adults	12,803	HR: 0.91 (0.77–1.07)

Regarding relative measures, boldface type indicates a statistically significant association (reference group: non-coeliac control subjects). All studies controlled for potential confounding factors. CeD: coeliac disease; CI: confidence interval; CIF: cumulative incidence function; HR: hazard ratio; SMR: standardized mortality rate.

2.5 Prothrombotic alterations in coeliac disease

Virchow had proposed that main events needed for thrombus formation include endothelial injury, hypercoagulability and blood flow abnormalities. In CeD, theoretically, the fragile balance of pro- and antithrombotic factors can be disturbed in many ways (Fig 1)[27-33].

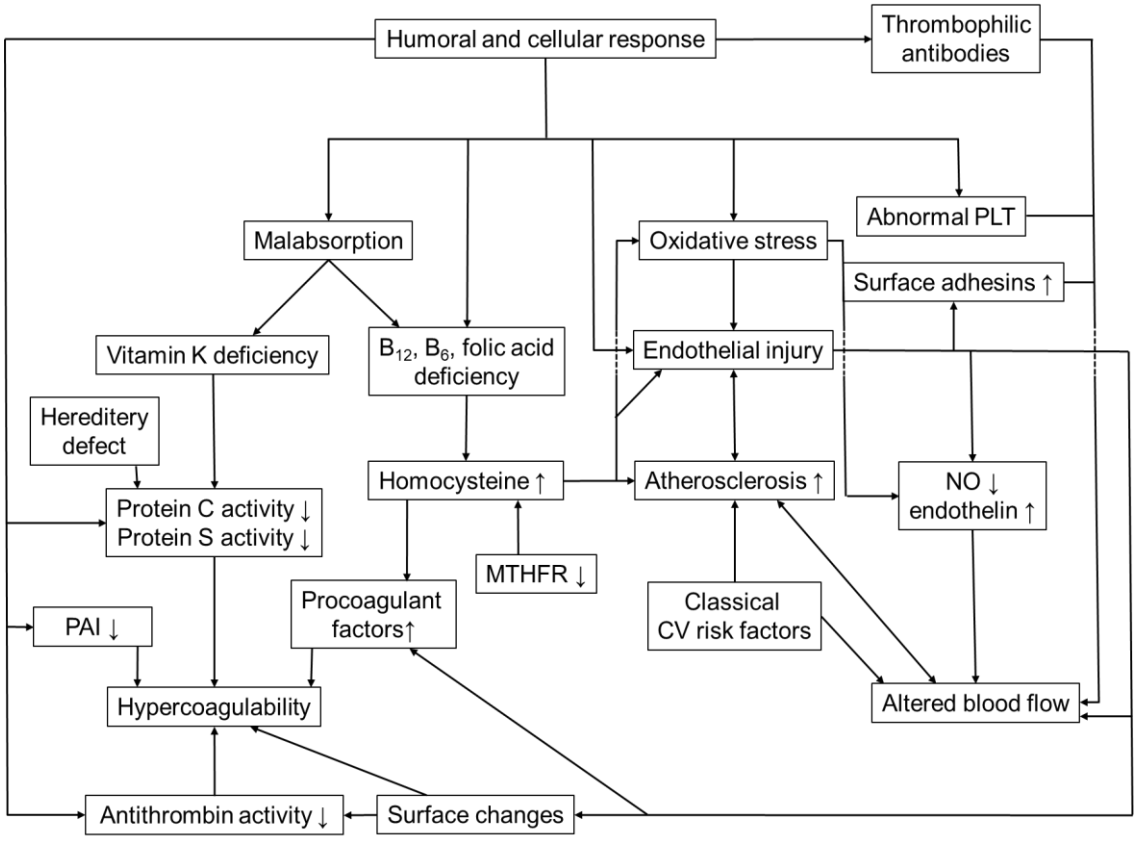

Fig 1. Model for the interplay of prothrombotic alterations in coeliac disease. CV: cardiovascular; MTHFR: methylene tetrahydrofolate reductase; NO: nitrogen oxide; PAI: plasminogen activator inhibitor; PLT: platelet. The figure is the author's own work.

2.6 Haemorheology

The definition of rheology reads as *"Rheology is the scientific field that deals with the flow and deformation behavior of materials,..."* in the paper of Baskurt and Meiselman[34]. Within the scope of rheology, haemorheology deals with the rheological properties of blood and its cellular elements[34-36].

In rheology, blood is a two-phase liquid or a solid-liquid suspension, where the formed elements serve as solid components. An essential term in rheology is viscosity, defined as the intrinsic property of fluids that indicates resistance to flow. Whole blood viscosity (WBV) is the most important in vivo rheological parameter, of which haematocrit is the primary determinant. Plasma viscosity (PV) falls between 1.10–1.35 mPa·sec at 37°C and is mainly determined by the macromolecule content of the plasma (primarily, by fibrinogen).

Red blood cells (RBC), the most abundant cells in the blood, give the majority of haematocrit, thereby being the primary determinants of WBV. Not only the quantitative but also the qualitative properties of RBCs are important in haemorheology. RBCs tend to deform and aggregate, described with erythrocyte deformability (ED) and erythrocyte aggregation (EA), respectively.

The unique structure (biconcave in shape) attributes unique mechanical properties to the cells. RBCs act like fluid droplets in the bloodstream, behave as elastic bodies and respond with reversible deformation to forces. ED is influenced by the properties of the cell membrane with the attached cytoskeletal protein frame underneath and by the intracytoplasmic viscosity. In addition to structural factors, energy-dependent processes, e.g. the intracellular Ca^{2+} concentration, affect mechanical properties as well. Intracytoplasmic viscosity is determined by haemoglobin. ED is quantifiable with the elongation index (EI). RBCs tend to arrange like a stack of coins, termed as rouleaux, in autologous plasma at rest. Aggregates readily dissolve to forces. Macromolecules of the plasma and that of RBCs' surface are involved in EA. Since macromolecules are essential regarding PV, determinants of EA partly overlap that of PV.

ED is important in large vessels as RBCs take parachute-like shape in the mainstream to reduce flow resistance. Likewise, the higher haematocrit in the mainstream promotes rouleaux formation, which has to disaggregate to allow RBCs to enter smaller vessels quickly. In the microcirculation, deformation is a must to pass through narrow capillaries quickly enough; besides, aggregates can plug capillaries so that resistance to flow increases.

Haemorheological alterations are present in many immune-mediated disorders including systemic lupus erythematosus[37], rheumatoid arthritis[37,38] and systemic sclerosis[39]. In gastroenterology, studies extensively investigated haemorheology in inflammatory bowel disease and reported prominent—sometimes, activity-dependent—prothrombotic alterations of fibrinogen, PV, ED and EA[40-42]. To our knowledge, no studies investigated haemorheological parameters in CeD.

3. Objectives and hypotheses

We designed this study to test if prothrombotic alterations characterize CeD patients compared to control subjects. Hypothesis testing aimed to add pieces to or subtract pieces from the puzzle of the complex system determining thrombus formation in CeD (see, Fig 1). Findings can explain, at least partly, the elevated CV risk and, in theory, can contribute to the excess in mortality of CeD. Besides, they can serve as the basis of future CV prevention studies by identifying or excluding potential pharmacological targets.

3.1 Primary objective and hypothesis

We aimed to test if alterations in haemorheological profile characterize CeD patients compared to non-CeD control subjects. Based on the literature data on multiple immune-mediated disorders, we hypothesize that haemorheological alterations are present in CeD patients.

3.2 Secondary objectives and hypotheses

- **We aimed to test if alterations in natural anticoagulant profile characterize CeD patients compared to non-CeD control subjects.** Based on case reports and the reasonable pathophysiological consequences of vitamin K deficiency, we expect lower natural anticoagulant activity in CeD patients compared to control subjects.
- **We aimed to assess if adherence to a GFD is associated with haemorheological alterations and natural anticoagulant activity in CeD.** As a GFD mitigates chronic inflammation and improves malabsorption, we expect less prominent (or no) alterations in CeD patients with strict adherence compared to those with poor adherence. We also hypothesize that the haemorheological and natural anticoagulant profiles of CeD patients with strict adherence are similar to those of control subjects so that the recovery on an adequate GFD is complete.
- **We aimed to test if the level of homocysteine is altered in CeD patients compared to non-CeD control subjects and to assess if dietary adherence affects the level of homocysteine.** Based on the literature data, we expect a higher level of homocysteine in CeD patients compared to control subjects, and in CeD patients with poor dietary adherence compared to those with good dietary adherence.

4. Methods

4.1 Ethics, pre-study protocol, study design and setting

The protocol of the study was permitted by the Regional and Local Research Ethics Committee (University of Pécs, Pécs, Hungary) under Reference Number 6917. To avoid reporting bias from occurring, we registered the study protocol onto the ISRCTN Registry (available online under registration number ISRCTN49677481) and published it in the BMJ Open[43] before starting the recruitment.

This study is a single-centre, observational study with case-control design, which recruited CeD patients and non-CeD control subjects prospectively. After providing standard care, we screened all patients attending the outpatient clinic of a tertiary centre, the Division of Gastroenterology, First Department of Medicine, University of Pécs (Pécs, Hungary) for eligibility over 12 months from Jun 2018 to May 2019.

4.2 Eligibility criteria

All participants had to provide signed informed consent, be ≥18 years of age, and subjected for blood collection independently of the study. CeD patients were newly diagnosed or followed cases, the diagnosis had to be histology-confirmed; otherwise, the criteria of the evidence-based guidelines[11,12] had to be fulfilled. In control subjects, CeD had to be excluded during a gluten-containing diet. All participants had to be free from advanced chronic conditions and any acute diseases or invasive procedures within 2 weeks of recruitment; other exclusion criteria are detailed in the pre-study protocol.

4.3 Flow and timing, questionnaires and measurements

After obtaining informed consent, we collected clinical data and biological samples. During data collection, we assessed baseline characteristics (including complete past medical history and medications) by detailed questioning complemented with the revision of medical files. Then, we completed the thrombophilia questionnaire. The visit ended with a dietary interview, followed by urine and blood collection.

Items of the thrombophilia questionnaire were selected based on two review papers[44,45] and were chosen to cover both arterial and venous risk factors of thrombus formation.

Laboratory measurements were performed after overnight fasting from venous blood. Measurements included routine laboratory parameters (including LDL, high-density lipoprotein [HDL] and total cholesterol); immunological indicators: CeD-specific antibodies (TGA IgA/G, EMA IgA) and antiphospholipid antibodies; haemostatic parameters (fibrinogen;

the activity of antithrombin, protein C, and protein S); homocysteine; and haemorheological parameters (for details, see Table 2).

Haemorheological measurements were performed in line with the recommendations of the International Expert Panel for Standardization of Haemorheological Methods[46].

We measured urine-GIP with a point-of-care test (iVYCHECK GIP Urine, Biomedal, Spain), an immunochromatographic dipstick containing G12 monoclonal antibodies targeted against 33-mer GIP.

4.4 Dietary adherence

Adherence to a GFD was estimated with (1) urine-GIP detection, (2) dietary review through interview by a trained dietician—assessed on a Likert-based visual analogous scale between 1 and 10 representing a regular gluten-containing diet and a theoretically perfect GFD, respectively—and with (3) CeD-specific antibodies (TGA-IgA, -IgG, EMA-IgA). We suspected gluten intake if the patient tested positive for urine-GIPs or any CeD-specific antibody as per the tests' manual of use, or the patient scored <8 based on dietary review.

Table 2. Haemorheology-related terms and measurements

Parameter	Measurement (unit)	Definition	Direction of unfavourable alteration*
Erythrocyte deformability	Elongation index (no unit)	change in the shape of red blood cells at high (3–30 Pa) and low shear stresses (0.3–1.69 Pa) (measured at nine shears in this study)	↓
Erythrocyte aggregation	Aggregation index (%)	integral in the change of light intensity 10 sec after disaggregation	↑
	Aggregation half-time (sec, symbol: $t_{1/2}$)	the time required for achieving half of the maximal aggregation after disaggregation	↓
	Threshold shear rate (1/sec, symbol: γ)	lowest shear that can maintain complete disaggregation	↑
Viscosity	Whole blood viscosity (mPa·sec)	an intrinsic property of fluid related to the internal friction of adjacent fluid layers sliding past one another (i.e., the measure of a fluid's resistance to flow) (measured at 90 1/sec in this study)	↑
	Plasma viscosity (mPa·sec)		↑
Plasma fibrinogen (g/L)		coagulation factor I, the precursor protein of fibrin	↑
Haematocrit (%)		the fraction of cellular components in the whole blood	↑

*regarding thrombus formation.

4.5 Outcomes

The primary outcomes included haemorheological parameters including ED, EA, WBV, PV, haematocrit and fibrinogen. The secondary outcomes included the activity of natural anticoagulants, including protein C, protein S and antithrombin; and the level of homocysteine.

4.6 Data management, sample size and statistical analysis

De-identified data were collected onto paper-based case report forms; data quality was ensured with 4-level quality control.

We planned the study to be a two-phase study. We recruited 50 CeD patients and matched control subjects in the first phase to determine further target numbers for the second phase. Completing the first phase, we realised that, to reach the level of significance for the observed mean differences between CeD and control subjects at $\alpha=0.05$ and $\beta=0.80$, we should recruit an unfeasible number of subjects, so that we decided to stop the study for lack of feasibility.

After matching by age (±5 years tolerance) and sex (±10% tolerance) in 1:1 ratio, categorical variables were given in proportions (% of total) and continuous variables were given with central tendencies (mean or median) and measure of dispersion (standard deviation, quartiles or range).

In univariate analysis, we used the Welch, Mann-Whitney, Chi^2- and Fisher's tests when comparing CeD patients to control subjects ($\alpha=0.05$); and one-way Analysis of Variance with the Tukey posthoc test or the Kruskal-Wallis test with the Mann-Whitney posthoc test (and Bonferonni correction) when comparing three groups—CeD patients with strict dietary adherence vs those with poor dietary adherence vs control subjects.

In multivariate analysis, we used the random forest method to determine the relative importance of each predictor and to display the important predictors graphically.

The calculations were carried out with IBM SPSS for Windows (version 25.0 statistical software package; Armonk, NY: IBM Corporation) and R statistical language (version 3.6, party statistical software package; R Core Team, Vienna, Austria).

5. Results

5.1 Characteristics of the participants included

We screened a total of 162 subjects for eligibility, 126 of which were eligible for inclusion. After matching, data of 50 CeD patients and 50 control subjects were analysed.

We did not find any significant difference in age, sex and other clinical characteristics between CeD patients and matched control subjects (Table 3). CeD patients had significantly lower levels of total, non-HDL-, LDL- and HDL-cholesterol; and higher eosinophil counts and RBC distribution width, compared to control subjects; whereas the groups were similar in laboratory parameters otherwise (Table 4).

CeD patients were, on average, 31.9 years old (range 0–73 years) at diagnosis. Three patients had not started a GFD at the time of the study, and all the others were ≥1 year on a GFD (median 5.5 years, range: 0.0–36.0 years). Six patients (12% of the total) tested positive for urine-GIP, 10 patients (20% of the total) scored <8 points on dietary review through interview (with median 9 points) and 14 patients (28% of the total) tested positive for TGA or EMA.

Control subjects attended a regular check-up (n=18), were admitted for investigation (n=16) or regular and mandatory occupational health assessment (n=16). All were on a gluten-containing diet and tested negative for CeD-specific antibodies.

Table 3. Clinical characteristics of the study population

	Coeliac group (n=50)	**Control group (n=50)**
Age at enrolment (mean; median [min–max] in years)	40.0; 40.0 [18.0–75.0]	40.4; 41.0 [19.0–74.0]
Females (n, %)	33 (66.0)	37 (74.0)
Venous thrombotic event in the history (n, %)	0 (0.0)	1 (2.0)
Arterial thrombotic event in the history (n, %)	1 (2.0)	0 (0.0)
Any thrombotic event in first-degree relatives (n, %)	14 (28.0)	12 (24.0)
Current smoker (n, %)	9 (18.0)	6 (12.0)
Alcohol consumption (≥7 units/week) (n, %)	3 (6.0)	4 (8.0)
Body mass index (mean; median [min-max] in kg/m^2)	23.6; 23.0 [16.4–40.5]	24.1; 23.7 [18.0–39.2]
Hypertension (n, %)	13 (26.0)	12 (24.0)
Peripheral arterial disease (n, %)	0 (0.0)	1 (2.0)
Type 2 diabetes mellitus (n, %)	3 (6.0)	3 (6.0)
Surgery (≤1 year) (n, %)	7 (14.0)	7 (14.0)
Immobilization (≤14 days) (n, %)	1 (2.0)	0 (0.0)
Travel by plane, bus or car ≥6 hours continuously ≤14 days (n, %)	3 (6.0)	5 (10.0)
Oral contraceptives (n, % of females)	8 (24.2)	10 (27.0)

Statistical comparison was not performed if the event number was ≤1 for categorical variables, p≥0.05 for all comparisons otherwise.

Table 4. Biochemical characteristics of the study population

	Coeliac group (n=50)	Control group (n=50)	p-value
Total cholesterol (mmol/L)	4.55; 4.45 [2.70–6.60]	5.32; 5.05 [3.20–9.30]	**0.001**
HDL-cholesterol (mmol/L)	1.43; 1.39 [0.65–2.97]	1.70; 1.63 [0.77–3.09]	**0.006**
LDL-cholesterol (mmol/L)	2.98; 3.02 [1.13–4.97]	3.50; 3.30 [0.45–7.85]	**0.015**
Non-HDL-cholesterol (mmol/L)	3.14; 3.14 [1.13–5.27]	3.61; 3.42 [1.98–7.67]	**0.032**
Triglyceride (mmol/L)	1.47; 1.31 [0.46–3.74]	1.69; 1.32 [0.60–7.18]	0.456*
Ultrasensitive CRP (mg/L)	3.4; 1.8 [0.0–23.5]	2.3; 1.4 [0.0–10.1]	0.342*
ESR (mm/h)	9.0; 4.0 [1.0–46.0]	6.1; 5.0 [1.0–27.0]	0.808*
Prothrombin time (sec)	11.4; 11.3 [9.6–14.0]	11.2; 11.2 [9.8–13.2]	0.252
Thrombin time (sec)	14.5; 14.6 [12.6–16.9]	14.3; 14.2 [12.0–17.4]	0.213
APTI (sec)	28.6; 28.3 [23.3–36.1]	30.1; 28.7 [19.0–72.9]	0.176
INR (no unit)	0.99; 0.98 [0.84–1.23]	0.98; 0.97 [0.86–1.09]	0.460
White blood cells (G/L)	7.3; 6.8 [3.7–16.2]	6.7; 6.4 [4.1–12.4]	0.165
Neutrophil granulocytes (G/L)	4.4; 4.2 [1.7–13.4]	3.9; 3.6 [1.8–9.2]	0.134
Lymphocytes (G/L)	2.1; 2.0 [1.2–4.2]	2.1; 2.1 [1.1–3.9]	0.947
Monocytes (G/L)	0.52; 0.48 [0.15–1.14]	0.50; 0.47 [0.18–0.99]	0.469
Eosinophil granulocytes (G/L)	0.18; 0.14 [0.01–0.60]	0.11; 0.10 [0.00–0.47]	**0.003**
Basophil granulocytes (G/L)	0.05; 0.04 [0.02–0.15]	0.05; 0.04 [0.01–0.09]	0.659
Red blood cells (T/L)	4.8; 4.8 [3.8–5.8]	4.9; 4.8 [3.9–5.8]	0.441
Haemoglobin (g/L)	140; 138 [99–169]	144; 142 [115–176]	0.149
MCV (fL)	84.8; 84.8 [65.6–97.6]	85.3; 85.3 [70.3–94.8]	0.603
MCH (pg)	29.0; 29.4 [20.3–32.8]	29.5; 29.7 [22.9–33.4]	0.252
MCHC (g/L)	343; 343 [309–361]	346; 346 [321–364]	0.118
RDW (%CV)	13.3; 12.6 [11.8–19.8]	12.6; 12.6 [11.0–14.5]	**0.022**
Platelets (G/L)	297; 283 [179–601]	282; 277 [126–432]	0.309
Vitamin B_{12} (ng/L)	450; 450 [156–785]	396; 424 [192–613]	0.076*

Regarding p-values, boldface type indicates a statistically significant difference. P-values labelled with asterisks (*) were generated with the Mann-Whitney test; all the other values were generated with the Welch test. [a]Based on protein electrophoresis, paraproteins were not present in any subject. Values are given in the following format: mean; median [min–max]. Missing data due to unsuccessful measurement(s): blood counts–one CeD patient, erythrocyte sedimentation rate–two CeD patients, coagulation parameters–one CeD patient and one control subject; vitamin B_{12} levels–three CeD patients and seven control subjects. APTI: activated partial thromboplastin time; CeD: coeliac disease; CRP: C-reactive protein; CW: coefficient of variation; ESR: erythrocyte sedimentation rate; HDL: high-density lipoprotein; INR: international normalised ratio; LDL: low-density lipoprotein; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; RDW: red blood cell distribution width.

5.2 Coeliac patients vs control subjects

5.2.1 Haemorheological parameters

EI at all shear stresses was lower in CeD patients compared to control subjects; however, the level of statistical significance was attained only at high shear stresses between 3–30 Pa (for the ektacytogram, see Fig 2); implying an impaired ED in CeD. Random forest analysis confirmed that CeD is an important predictor of EI at high shear stresses (Fig 3) but not at low shear stresses.

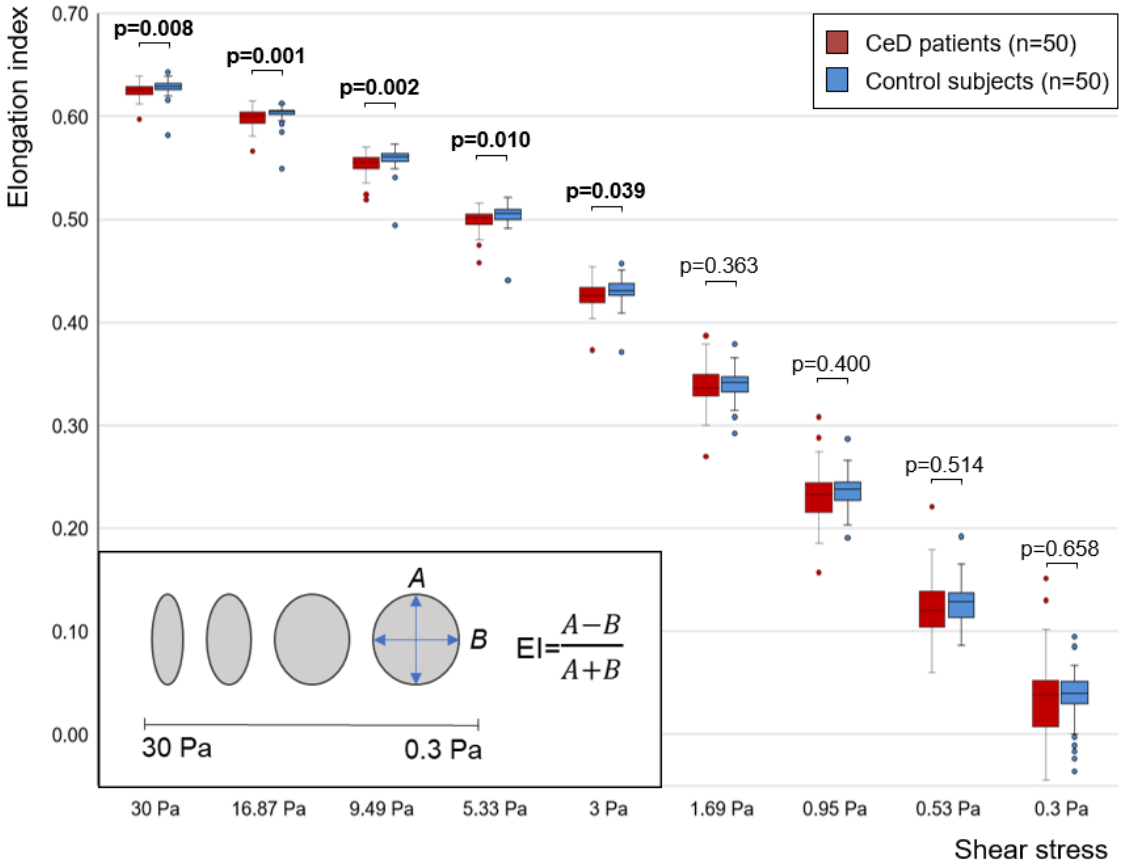

Fig 2. Erythrocyte deformability at different shear stresses (ektacytogram) in coeliac patients vs control subjects. The horizontal axis shows different shear stresses from 0.3 to 30 Pa; the vertical axis shows the elongation index. P-values were generated with the Mann-Whitney test; boldface type indicates a statistically significant difference. The number of participants is 100 in the analysis. **Inlet:** A model for erythrocyte deformation at different shear stresses describing the transition from biconcave to ellipsoid shape. In the equation, EI stands for the elongation index, A and B represent the long and short axes of the red blood cells, respectively; as indicated with the arrows. CeD: coeliac disease. The figure is the author's own work.

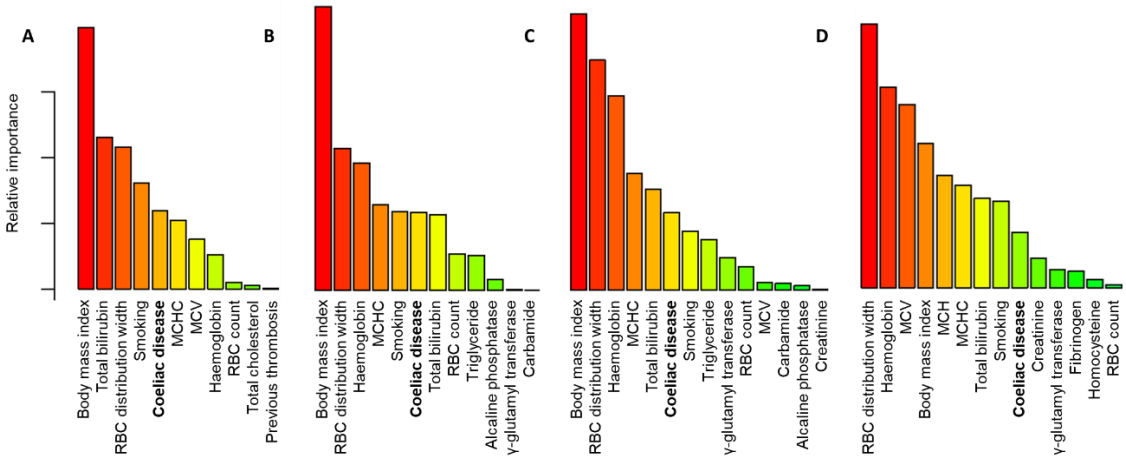

Fig 3. Important predictors of erythrocyte deformability represented by the elongation index at different shear stresses. Panels A, B, C and D show elongation indices at 30, 16.87, 9.49 and 5.33 Pa, respectively. The figures were generated with random forest analysis. We added 34 co-variates to the model, but only the important predictors of the outcome are shown. The relative importance is proportional to the height of the bars. The number of participants is 97 in the analysis. MCH: mean corpuscular haemoglobin; MCHC mean corpuscular haemoglobin concentration; MCV mean corpuscular volume; RBC: red blood cell. The figure is the author's own work.

There was no statistically significant difference in other haemorheological parameters (i.e. haematocrit, the level of fibrinogen, the parameters describing EA, WBV and PV) between the groups (Table 5). In line with these, random forest analysis did not highlight CeD as an important predictor of any of these outcomes.

5.2.2 Natural anticoagulants and homocysteine

Although numerical values were lower in CeD, we observed no statistically significant difference in the activity of protein C, protein S and antithrombin between the groups (Table 6). In line with these, random forest analysis did not highlight CeD as an important predictor of any of these outcomes.

CeD patients had a significantly higher level of homocysteine (median 9.0 μmol/L with range 5.1–13.7 μmol/L vs median 8.7 μmol/L with range 4.4–42.9 μmol/L for CeD and control groups, respectively; p=0.040). Random forest analysis did not highlight CeD as an independent predictor of the level of homocysteine.

Table 5. Haemorheological parameters in coeliac patients vs control subjects

	Coeliac group (n=50)	**Control group (n=50)**	**p-value**
Haematocrit (%)	43.3 ± 3.6	44.4 ± 3.3	0.117
Whole blood viscosity (mPa·sec)	4.04 ± 0.43	4.14 ± 0.43	0.347
Plasma viscosity (mPa·sec)	1.24 ± 0.16	1.27 ± 0.15	0.209
Fibrinogen (g/L)	2.90 [2.59–3.70]	3.16 [2.71–3.59]	0.948*
Erythrocyte aggregation			
AI (%)	63.8 ± 10.0	64.6 ± 6.3	0.613
$T_{1/2}$ (sec)	2.31 ± 1.35	2.06 ± 0.71	0.677
γ (1/sec)	106.9 ± 50.0	102.3 ± 29.6	0.951

Results on erythrocyte deformability are shown in Fig 2. The p-values labelled with an asterisk (*) were generated with the Mann-Whitney test, other p-values were generated with the Welch test after logarithmic transformation in the case of whole blood viscosity, plasma viscosity, $t_{1/2}$, and γ. Value are given in mean ± standard deviation except for fibrinogen given in median ± quartiles. AI: aggregation index.

Table 6. Natural anticoagulants in coeliac patients vs control subjects

	Coeliac group (n=50)	**Control group (n=50)**	**p-value**
Antithrombin activity (%)	120.28 ± 14.39	121.84 ± 14.16	0.589
Protein C activity (%)	124.60 ± 33.72	134.30 ± 32.47	0.146
Protein S activity (%)	98.40 ± 29.44	104.02 ± 30.35	0.338

Values are given in mean ± standard deviation. P-values were generated with the Welch test after logarithmic transformation in the case of activity of antithrombin and protein S. Data were missing due to unsuccessful measurement(s) in the case of one CeD patient and one control subject.

5.3 The effects of dietary adherence

5.3.1 Haemorheological parameters

ED at high shear stresses was impaired both in CeD patients who were seropositive and seronegative (Fig 4A, solid green arrows) and both in CeD patients with poor and good dietary review through interview (Fig 4B, solid green arrows) compared to control subjects. However, ED at high shear stresses did not differ significantly between seropositive and seronegative CeD (Fig 4A, yellow dash line) or urine-GIP+ and urine-GIP− CeD patients (Fig 4C, yellow dash line), whereas CeD patients with good dietary review through interview had better ED only at EI_{30Pa} compared to those with poor adherence (Fig 4B, blue dash arrow). These suggest that the impairment in ED at high shear stresses is independent of the EMA/TGA-mediated immune response and only partly dependent on dietary adherence. Interestingly, ED at low shear stresses did not differ across groups irrespective of seropositivity and dietary review through interview (Figs 4D and 4E, yellow dash line). In contrast, urine-GIP+ CeD patients had significantly impaired ED compared to urine-GIP− CeD patients and control subjects (Fig 4F, solid green arrow), without a significant difference between urine-GIP− CeD patients and controls (Fig 4F, yellow dash line). These results suggest that ED at low shear stresses may not be influenced by EMA/TGA-mediated immune response but may be influenced by other effects of gluten or related pro-inflammatory reaction.

EA seemed to be significantly impaired in CeD patients with poor dietary review through interview compared to those with good results and control subjects. The association applies to aggregation index (Fig 5A), aggregation half-time (Fig 5B) and threshold shear rate consistently (adjusted p-values <0.01 for all), suggesting prothrombotic alterations in CeD patients with poor adherence. However, seropositive CeD patients did not differ from seronegative ones so that the EMA/TGA-mediated immune response unlikely explains the findings.

Although WBV was lower in CeD patients with good dietary review through interview compared to CeD patients with poor results, neither differed significantly from control subjects. Haematocrit, WBV and PV did not seem to be different substantially across the groups.

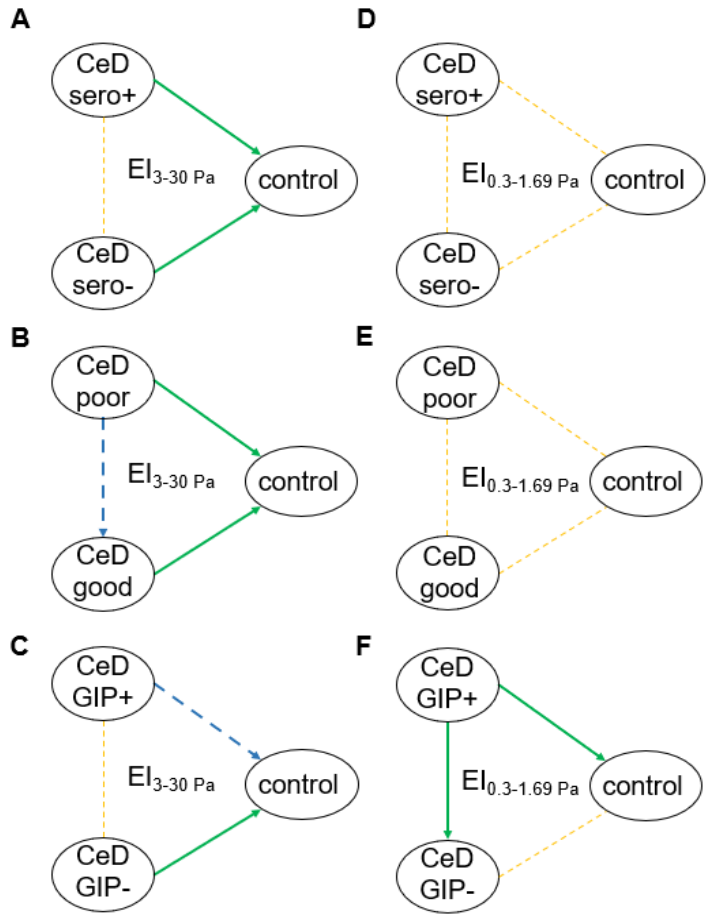

Fig 4. Model for the association of erythrocyte deformability with dietary adherence. Erythrocyte deformability is represented by the elongation index. **A** and **D**: coeliac-specific serology and erythrocyte deformability at high and low shear stresses, respectively. **B** and **E**: dietary review through interview and erythrocyte deformability at high and low shear stresses, respectively. **C** and **F**: urine-GIP detection and erythrocyte deformability at high and low shear stresses, respectively. P-values were adjusted for multiplicity. Green solid lines indicate $p<0.05$ at 3–5 shear stresses favouring the group at the arrow tip. Blue dashed lines indicate $p<0.05$ at one shear stress favouring the group at the arrow tip. Yellow dashed lines indicate no significant difference between groups. CeD: coeliac disease; GIP: gluten immunogenic peptide. The figure is the author's own work.

5.3.2 Natural anticoagulants and homocysteine

Dividing patients by seropositivity, dietary review through interview or urine-GIP measurement did not reveal any difference across the groups. In line with this, random forest analysis did not highlight any modality estimating dietary adherence as an important predictor of the outcomes.

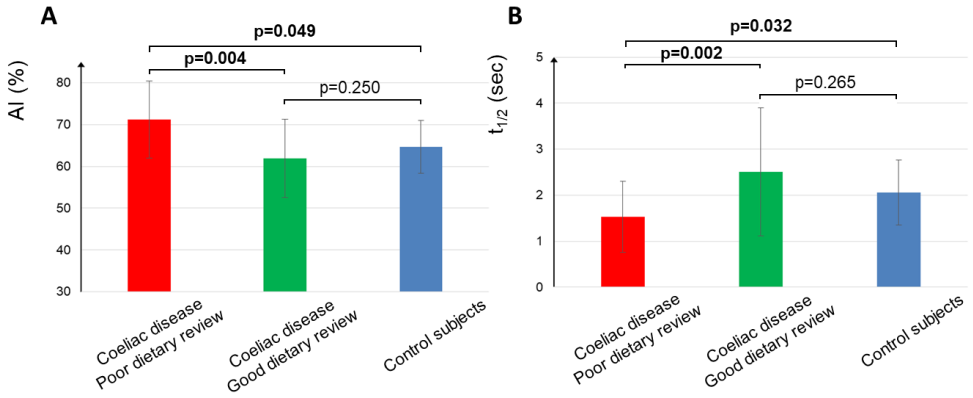

Fig 5. Erythrocyte aggregation and dietary review through interview. A: Aggregation index across groups (p for interaction: 0.005). **B:** Aggregation half-time across groups (p for interaction: 0.003). P-values were generated with the one-way Analysis of Variance model after logarithmic transformation of the data in the case of $t_{1/2}$, and were adjusted for multiplicity; boldface type indicates a statistically significant difference. The number of patients is 100 in the analysis. $t_{1/2}$: aggregation half-time; AI: aggregation index. The figure is the author's own work.

6. Discussion

6.1 Summary of findings

This study aimed to assess the haemorheological and natural anticoagulant profiles of CeD patients compared to non-CeD control subjects. We found an impaired ED at high shears in CeD, but ED at low shears, other haemorheological parameters and the activity of natural anticoagulants did not differ significantly between the groups. Besides, the level of homocysteine was significantly higher among CeD patients compared to control subjects. When we investigated the associations of dietary adherence with the outcomes, alterations in ED proved to be rather gluten-independent, whereas alterations in EA and WBV were gluten-dependent. As, based on the literature data, haemorheological alterations and hyperhomocysteinaemia are associated with CV events, our findings call the attention for CV prevention in CeD patients and highlight the importance of a strict, lifelong GFD.

6.2 Explanation and elaboration

6.2.1 Haemorheological parameters and cardiovascular involvement

Deformation is required for RBCs to pass through narrow capillaries as well as to take parachute-like shape, reducing resistance to flow in the mainstream of the large arteries. If ED is severely impaired—simply said, RBCs become rigid—microcirculation deteriorates, viscosity increases, causing a tendency for thrombus formation. The higher the EI, the more significant the change in shape, so that the better the deformability. Although the exact cause of the impaired ED in CeD is yet to be discovered, we can propose mutually non-exclusive theories. (1) In functional hyposplenism, seen in 16–77% of CeD patients[47], the spleen no longer removes old RBCs from the circulation. (2) Increased oxidative stress and reduced nitrogen oxide production can impair ED. (3) The greater the variation in RBC distribution width, the lower the EI[48]. (4) Immune-mediated comorbidities, known to impair ED, occur more frequently in CeD.

The unwanted effect of the impaired ED likely manifests via the deterioration of the microcirculation and, in severe cases, via an increase in WBV. Since changes in ED in CeD seem rather diet-independent, efforts to restore normal ED (and to prevent further decline) with interventions other than a GFD may be considered. Current nutritional trends show an unfavourable nutrient profile of a GFD regarding the development of CV risk factors, particularly obesity, known to be an independent predictor of EI[49]. In addition to regular exercise, preventive strategies should focus on education to improve nutritional awareness to optimize body weight. The efficacy of statins has been proven in diabetes mellitus and

dyslipidaemia as well as the pharmacological control of hypertension[50] and diabetes mellitus[51], comorbidities impairing ED. Besides, the effects of many other nutritional supplements, e.g. resveratrol[52], are promising.

EA is a physiological phenomenon until the dynamic balance of aggregation and disaggregation is maintained. Increased EA halts flow in the microcirculation and increases WBV. Although CeD patients did not significantly differ from control subjects in any of these parameters, CeD patients with poor dietary review through interview showed an increased tendency for EA compared to patients with good adherence and control subjects, having no difference between the latter two groups. To conclude, good dietary adherence restores normal EA during a GFD. The increased EA can be manifested in elevated WBV in patients with poor dietary adherence, as measured in our study as well. The alteration of WBV, the most important haemorheological parameter, has far-reaching consequences. The predictive role of WBV to CV events are extensively studied in a large variety of CV diseases. The reduction of the increased EA and WBV might have beneficial effects in CeD: a strict GFD is expected to mitigate or restore these alterations as they proved to be dependent on gluten intake in our study.

6.2.2 Natural anticoagulants and homocysteine

Natural anticoagulants are more important determinants of venous rather than arterial thrombotic events. Hereditary protein C, protein S and antithrombin deficiency increase the risk of venous thromboembolism to 7-fold, 5–11.5-fold and 2.2–8.1-fold, respectively[53]. Although the figures were lower with CeD compared to control subjects, we did not identify a significantly reduced activity of protein C and protein S with CeD. These findings are in line with the observation that a 1-year GFD recovers regular vitamin K status[54]; therefore, the synthesis of protein C and protein S normalizes. These findings corroborated with our observations on the activity of antithrombin.

An increase in the level of homocysteine is a robust and independent predictor of ischaemic stroke[55] and myocardial infarction.[56] CeD patients at diagnosis exhibit a higher level of homocysteine, compared to control subjects[28,31]. However, studies resulted in divergent results as to whether the level of homocysteine reduces[28] or continues to remain elevated[27,31] during a GFD. In our study, which recruited CeD patients dominantly on a GFD, we measured a significantly higher level of homocysteine in CeD patients compared to control subjects, but dietary adherence seemed not to affect it. Administration of vitamin B_6 and folate (maybe vitamin B_{12} as well) might improve hyperhomocysteinaemia.

6.3 Strengths and weaknesses

There are several strengths of the study, which worth being highlighted. (1) The novelty of the study roots in that—to the best of our knowledge—none have investigated the haemorheological and natural anticoagulant profiles of CeD patients in a controlled study yet. (2) The study relies on an exhaustively detailed pre-study protocol, which is freely accessible for all. (3) The data quality is 100% for the primary and almost 100% for secondary outcomes. (4) Matching by age and sex mitigated between-group differences in major co-variates. (5) Dietary adherence of CeD patients was approached multimodally, including state-of-the-art urine-GIP detection. (6) Haemorheological measurements were rigorously standardized. (7) A complex analytical approach was developed and executed by a skilled statistician.

Nevertheless, we must acknowledge that the study has several limitations. (1) A prospective cohort study could have served solid evidence on the changes in the outcomes during GFD. (2) Although we planned to measure the level of erythrocyte folate, we did not have access to the required resources. (3) Although all diagnoses of CeD were biopsy-verified, we included newly diagnosed and followed cases, which increased the clinical heterogeneity of the population. (4) The limited sample size risks β-type error. (5) The few pieces of missing data limited the use of multivariate analysis. (6) We did not achieve a perfect match for each case due to the limited sample size of the control group. (7) The random forest model quantifies the relative but not the absolute effect of the predictors.

6.4 Generalizability of the findings

The study population included relatively young—on average, 40 years of age at inclusion—CeD patients. Likely due to the strict eligibility criteria, both CeD patients and control subjects had few and relatively mild comorbidities. Besides, 47 of 50 CeD patients were on a GFD ≥1 year, which allowed time to recover from malabsorption and for the chronic inflammation to cool down. It is reasonable to assume, based on the literature data, that haemorheological alterations aggravate with ageing and due to the heavy burden of age- and lifestyle-related comorbidities, providing further ground for CV events to develop. Having in mind that the elderly diagnosis of CeD is becoming increasingly common, these alterations gain further attention.

7. Summary of novel findings and perspectives

1. This study confirmed that **ED at high shear stresses is impaired**, whereas ED at low shear stresses, parameters describing EA, PV, WBV, fibrinogen and haematocrit are not altered in CeD compared to control subjects (Fig 6). **CeD is an independent predictor of ED** in random forest analysis.
2. This study confirmed that **adherence to a GFD is**, at least party, **associated with haemorheological alterations in CeD**. Although the impaired ED seems to be mainly independent of dietary adherence, **poor dietary adherence is associated with prothrombotic alteration** in parameters describing EA and WBV.
3. This study did not confirm that the activity of natural anticoagulants is reduced in CeD patients compared to control subjects or in CeD patients with poor dietary adherence compared to those with good adherence.
4. This study confirmed **an elevated level of homocysteine**, not influenced by dietary adherence, in CeD patients compared to control subjects.

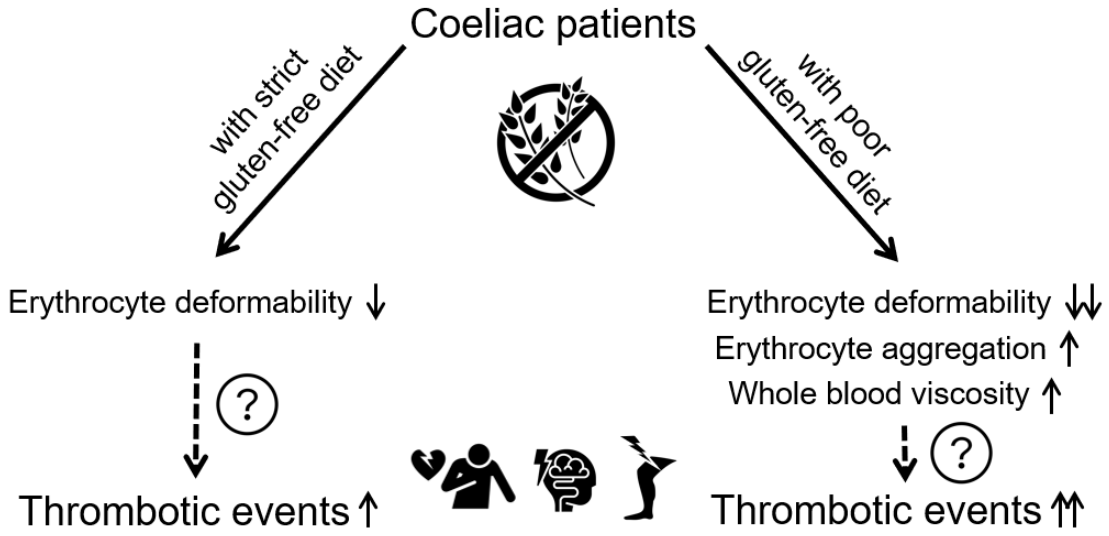

Fig 6. Summary of prothrombotic haemorheological changes among coeliac patients compared to control

Further studies are needed to verify our findings in a prospective cohort study recruiting cases immediately after diagnosis of CeD and repeating the measurements later during a GFD; and to test if pharmacological and non-pharmacological interventions, such as antioxidants, statins or lifestyle changes, can restore or improve ED in CeD.

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Finally, thanks to **my family, friends and colleagues** for the continuous support I have received. Without it, my thesis would have never been completed.

9. Scientometrics

Publications and metrics rely on the MTMT2, the data were extracted on 28th August 2020.

Scientific papers:

- Total: 48

Impact factor:

- First and last author: 33.797
- Cumulative: 145.912

Citations:

- Independent: 152
- Cumulative: 173
- Hirsh index: 7

List of publications

Papers upon which this thesis relies (n=2, cumulative impact factor: 6.464):

1. **Szakács Z**, Csiszár B, Kenyeres P, et al. Haemorheological and haemostatic alterations in coeliac disease and inflammatory bowel disease in comparison with non-coeliac, non-IBD subjects (HERMES): a case-control study protocol. *BMJ Open.* 2019;9(3):e026315 (**Q1, IF: 2.496**).
2. **Szakács Z**, Csiszár B, Nagy M, et al. Diet-dependent and diet-independent haemorheological alterations in celiac disease: A case-control study. *Clin Transl Gastroenterol.* 2020 (**Q1, IF: 3.968**) (article in press).

Papers loosely related to the topic of the thesis (n=5):

1. Bajor J, **Szakács Z**, Farkas N, et al. Classical celiac disease is more frequent with a double dose of HLA-DQB1*02: A systematic review with meta-analysis. *PLoS One.* 2019;14(2):e0212329-e0212329 (**Q1, IF: 2.740**).
2. Bajor J, **Szakács Z**, Juhász M, et al. HLA-DQ2 homozygosis increases tTGA levels at diagnosis but does not influence the clinical phenotype of coeliac disease: A multicentre study. *Int J Immunogenet.* 2019;46(2):74-81 (**Q3, IF: 1.130**).
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