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

PhD Thesis

Doctoral School of Pharmacological and Pharmaceutical Sciences

Head: Erika Pintér, MD, PhD, DSc

Translational Medicine Programme leader:

Péter Hegyi, MD, PhD, DSc, MAE

Zsolt Szakács, MD

Institute for Translational Medicine, Medical School, University of Pécs, Pécs, Hungary

Supervisor:

Judit Bajor, MD, PhD

First Department of Medicine, Medical School, University of Pécs, Pécs, Hungary

Pécs, 2020

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2. List of abbreviations

The list comprises only abbreviations used in the main text of the thesis (chapters 4−10). Abbreviations used in the Synoptics (chapter 3) are spelt out within the chapter, those used in tables and figures are spelt out in the corresponding footnotes.

AI: aggregation index
CeD: coeliac disease
CI: 95% confidence interval
CV: cardiovascular
EA: erythrocyte aggregation
ED: erythrocyte deformability
EI: elongation index
EMA: anti-endomysial antibody
GFD: gluten-free diet
GIP: gluten immunogenic peptide
HDL: high-density lipoprotein
HLA: human leukocyte antigen
LDL: low-density lipoprotein
LORCA: Laser-assisted Optical Rotational Cell Analyzer
PV: plasma viscosity
RBC: red blood cell
SMR: standardized mortality rate
TGA: anti-tissue transglutaminase antibody
VTE: venous thromboembolism
WBV: whole blood viscosity

3. Synoptics

Coeliac disease (CeD) is a systemic immune-mediated disease, which develops upon gluten ingestion in genetically predisposed individuals, and affects about 1% of the population worldwide. The overall mortality of CeD patients is two-fold compared to the global average. The excess mortality stems from a higher risk of lymphoproliferative and cardiovascular diseases, including acute arterial and venous thrombotic events. The pathophysiology of the promoted thrombus formation in CeD is multifaceted. The main suspected factors include hyperhomocysteinaemia, reduced activity of natural anti-coagulant proteins, endothelial injury, subclinical chronic inflammation, thrombophilic antibody formation, immune- and non-immune-mediated comorbid conditions and accelerated atherosclerosis. Besides, as seen in many immune-mediated diseases, alterations in rheological properties of blood and its cellular elements, i.e. haemorheological alterations, are presumed to be potential contributors of thrombus formation in CeD. In this study, we aimed to assess the haemorheological, natural anti-coagulant and homocysteine profiles of CeD patients to explore the background of the elevated cardiovascular risk and to suggest potential targets for prevention.

This study is a case-control study (registered under registration number ISRCTN49677481), in which we recruited patients from a gastroenterology outpatient clinic. Cases were adult, biopsy-verified CeD patients, controls were subjects in whom CeD was excluded based on clinical and laboratory assessment. All participants were free from acute and advanced chronic diseases.

After obtaining written consent, we recorded clinical characteristics, including risk factors of arterial and venous thrombus formation. In addition to a routine set of laboratory investigations, we measured haemorheological parameters, the activity of natural anticoagulants (protein C, protein S and antithrombin) and the level of homo-cysteine. As primary outcomes, the haemorheological profile included haematocrit, whole blood viscosity (WBV), plasma viscosity, fibrinogen, erythrocyte aggregation (EA, described with the aggregation index, aggregation half-time and threshold shear rate) and erythrocyte deformability (ED, described with the elongation index at high [between 3–30 Pa] and low shear stresses [0.3–1.69 Pa]). We compared CeD patients to control subjects in univariate analysis and performed multivariate analysis with the ran-dom forest model to identify independent predictors of the outcomes. In CeD patients, we estimated adherence to a gluten-free diet with CeD-specific serology (seronegative vs

seropositive), urine-gluten immunogenic peptide detection (positive vs negative) and with dietary review through interview (poor vs good). We divided CeD patients by dietary adherence and compared them to each other and to the control group.

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 was lower at all shear stresses in CeD compared to the control group, but the level of significance was attained only at high shear stresses; suggesting an impaired ED in CeD. In line with this, CeD proved to be an independent predictor of elongation index only at high shear stresses. Besides, CeD patients had a significantly higher level of fasting homocysteine compared to the control group (median 9.0 vs 8.7 μmol/L, respectively; p=0.040). The other outcome parameters were similar between the groups.

Seronegative CeD patients showed similar ED to seropositive ones and to control subjects, but a poor dietary review was associated with an impaired ED compared to a good dietary review. Consequently, the impairment in ED at high shear stresses seemed to be only partly dependent on dietary adherence. Parameters describing EA and WBV were consistently shifted towards a prothrombotic direction in CeD patients with a poor dietary review compared to those with a good dietary review; highlighting the importance of a strict gluten-free diet regarding EA and WBV. The other outcomes 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, CeD patients with poor dietary review have impaired EA and WBV, which seems to restore on a strict gluten-free diet. The other outcome parameters seem not to be altered in CeD. These diet-dependent and diet-independent prothrombotic changes can contribute the elevated cardiovascular risk in CeD, and raise the need for the introduction of preventive measures (e.g. weight control, dietary education, statins or polyphenols).

4. Introduction

Coeliac disease (CeD) is a systemic immune-mediated disease, which develops upon gluten ingestion in genetically predisposed individuals. Disease burden results from deterioration of quality of life with symptoms as well as from complications and co-morbid conditions. The treatment of CeD is the complete, lifelong exclusion of dietary gluten.

4.1 Prevalence and incidence of coeliac disease

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%)[1]. The prevalence varies across geographical regions: in contrast to 3% in Sweden[2], CeD is extremely rare, e.g., in Japan (0.05–0.19%)[3]. In Hungary, the prevalence of CeD is around the global average: 1.17–1.38%[4,5].

The incidence of CeD is increasing over time: the average annual increment was 7.5% (CI: 5.8–9.3%) over the past decades in a meta-analysis (Fig 1)[6]. The rise can be explained by improved diagnostics and better awareness of CeD and CeD-associated co-morbid conditions as well as by pathophysiological reasons[6] (e.g. the hygiene-hypothesis[7]).

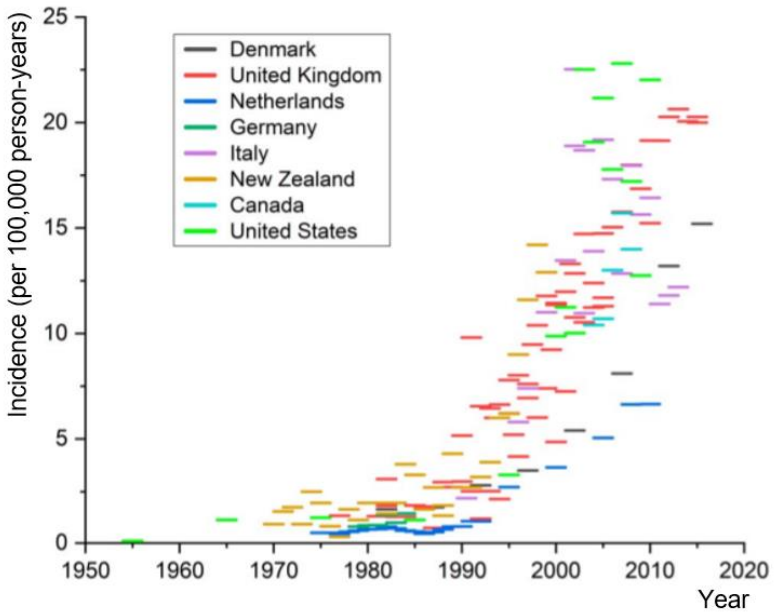


Fig 1. Incidence of coeliac disease over time per country. Each horizontal bar represents a study, the length of the bar covers the recruitment period. Reprinted from 'Incidence of Celiac Disease Is Increasing Over Time: A Systematic Review and Meta-analysis' by King et al., 2020, *Am J Gastroenterol*.

4.2 Pathomechanism of coeliac disease

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

4.2.1 Gluten

The protein content of wheat kernel (Fig 2) is 8–15%, the majority (85–90%) of which is given by gluten, a mixture of storage proteins[8]. Wheat gluten 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). The gluten content of the grains is a critical determinant of the dough quality of bread and other bakery products as gluten strings retain carbon-dioxide during leavening[8]. The chemical composition and quantity of gluten vary across plants, even within the same species depending on environmental factors (e.g. temperature during maturity)[8].

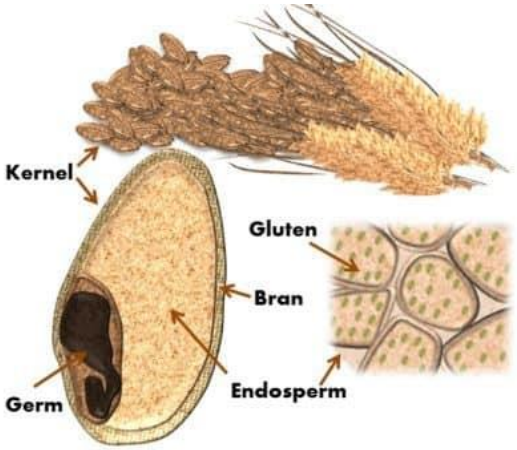


Fig 2. The anatomy of a grain of wheat. The endosperm contains gluten and starch. Downloaded from https://corehealthstl.com/.

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. Among GIPs, the 33-mer GIP (amino acid sequence: LQLQPFP(QPQLPYP)$_3$QPQPF) is considered to have the most potent immune-stimulatory property. Derivatives of gliadin (wheat), hordein (barley), secalin (rye) and, maybe, avenin (oats) are immunogenic, unlike the storage proteins of other plants being non-immunogenic in CeD patients[9]. In the followings of the thesis, the word, ‘gluten’ is used only for storage proteins containing immunogenic sequences.

Other grain proteins (e.g. glutenins, amylase-trypsin inhibitor, wheat germ agglutinins) were implicated to have immune- and non-immune-mediated harmful effects as well[8,10].

4.2.2 Genetics

Concordance of CeD reaches 75–86% among monozygotic twins[11], whereas first-degree relatives are at 10–15% risk of developing CeD[12]. The role of HLA class II heterodimers in the pathomechanism is extensively studied. Either HLA-DQ2 or -DQ8 has to be present in nearly all CeD patients[13]. HLA heterodimers comprise an α and a β subunit, the alleles of which are encoded on chromosome 6 (Fig 3)[14].

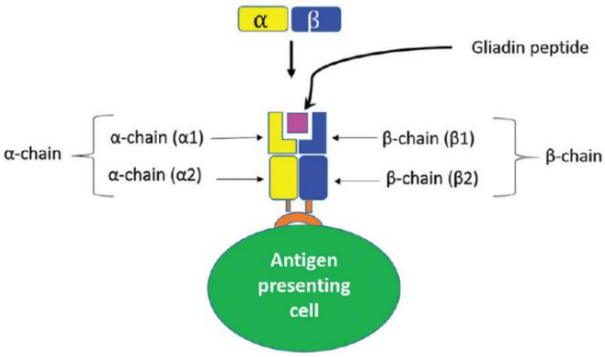


Fig 3. Model for the presentation of gluten immunogenic peptides on human leukocyte antigen. Reprinted and adapted from 'Genetic susceptibility for celiac disease is highly prevalent in the Saudi population' by Al-Hussaini, 2018, *Saudi J Gastroenterol*.

Depending on the allele constitution, serotypes (i.e. HLA-DQ2 or -DQ8) comprise several HLA haplotypes (e.g. HLA-DQ2.5 homozygote), each carries a different risk of CeD on a continuum ranging from low to high risk[15]. The alleles determine peptide-binding properties of a haplotype, those encoding the β chain seems to be the decisive ones over those encoding the α chain. Based on experimental data, the HLA-DQ2.5 homozygote haplotype—the most common one in CeD, which contains a double dose of HLA DQB1*0201 allele—is the most effective in antigen presentation[16]. The number of copies of B1*0201 strongly affects peptide-binding properties (the more copies, the higher the affinity), thereby modulating the risk of CeD[15] and, maybe, clinical phenotype[17,18].

CeD patients carry HLA-DQ2 in 90–95% and -DQ8 in 5–10%[19]. Contrasting the approximately 1% worldwide prevalence of CeD[1], one or both serotype occurs in 20–40% in the general population[20]. This one-magnitude difference means that other factors than HLA (and gluten) are required to elicit CeD. The missing chain(s) in disease development can be non-HLA-related genetic factors[21], but environmental exposures (e.g. rotavirus infection in childhood) were implicated as well[22].

4.2.3 Immunological response

Fig 4 displays a model for the immune-mediated pathogenesis of CeD, in which both the innate and the adaptive (Th_1-dominant) immune systems are involved. (The numbers in circles referenced in the following paragraph correspond to the numbers in Fig 4.) The model explains how alimentary gluten leads to intestinal mucosal damage and antibody formation in genetically susceptible individuals[10].

①After 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. ②Raised expression of zonulin, a protein increasing the permeability of the gut barrier, can be observed in CeD and facilitates the paracellular transport[23]. ③GIPs activate local antigen-presenting cells in the lamina propria and trigger interleukin synthesis, ④leading to further cellular activation. ⑤Meanwhile, GIPs gain negative charges through deamidation by tissue transglutaminase enzyme type 2, so that their affinity to bind HLA increases. ⑥Deamidated 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 (e.g. interferon-γ, tumour necrosis factor-α, interleukin-21), ⑦which activate cytotoxic T-, NK- and plasma cells. ⑧Intraepithelial lymphocytes—mainly the T-cells— and activated fibroblasts cause local mucosal damage, ending up in total villous atrophy, clinically manifesting in malabsorption. ⑨Besides, plasma cells start forming IgA-, IgG- and IgM-type 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[10].

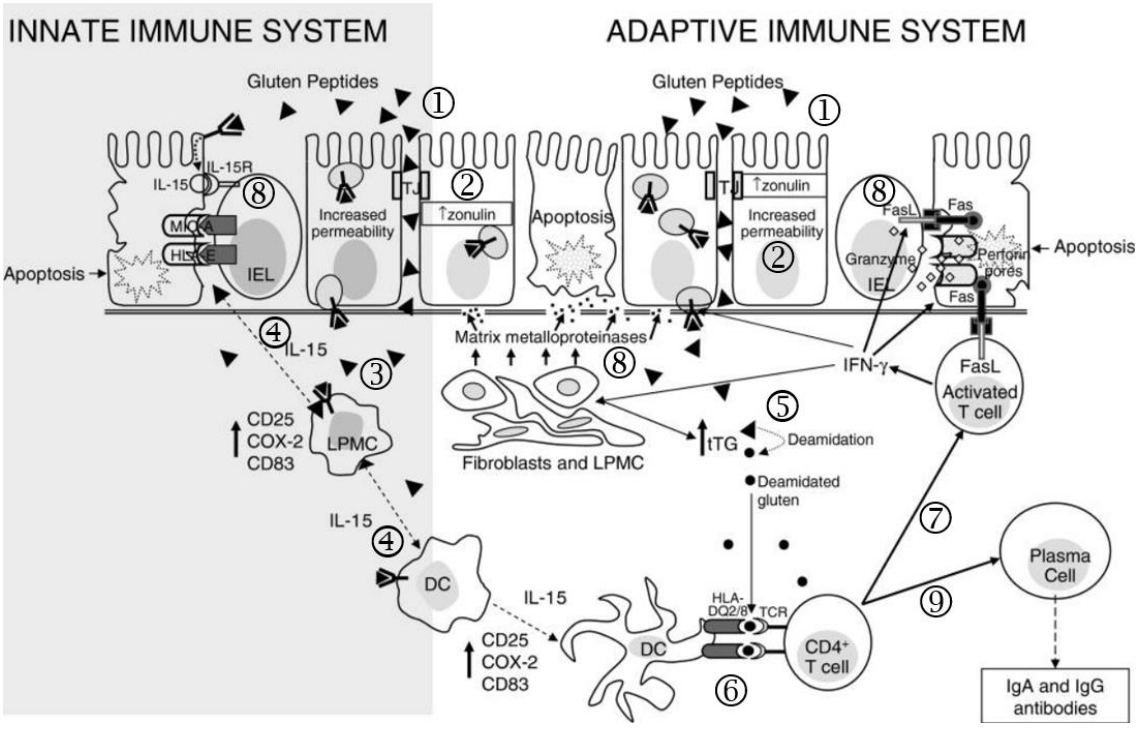


Fig 4. Model for the immune pathogenesis of coeliac disease. The numbers in circles are referenced in the text. DC: dendritic cell; IEL: intraepithelial lymphocyte; IL-15: interleukin-15; IL-15R: interleukin-15 receptor; IFN-γ: interferon-γ; LPMC: lamina propria mononuclear cell; tTG: tissue transglutaminase. Reprinted and adapted from 'The immune recognition of gluten in coeliac disease' by Ciccocioppo et al., 2005, *Clin Exp Immunol*.

4.3 Clinical characteristics and diagnosis of coeliac disease

The diagnosis of CeD relies on four pillars: signs and symptoms, serology, histology and genetic testing.

4.3.1 Signs and symptoms

Symptoms can be classified as gastrointestinal and extraintestinal or as 'typical' and 'atypical'. The 2012 Oslo consensus conference aimed to standardize the terminology used to describe clinical phenotype, based on which we differentiate classical, non-classical and asymptomatic CeD[24]. Patients with classical CeD present with signs and symptoms of malabsorption (diarrhoea, steatorrhoea, weight loss, growth failure, oedema due to hypalbuminaemia). In contrast, patients with non-classical CeD do not have malabsorption but have other symptoms or CeD-specific conditions (e.g. abdominal pain, constipation, vomiting, fatigue, dermatitis herpetiformis, vitamin or mineral deficiencies, infertility, osteoporosis, coeliac hepatitis). Patients with asymptomatic (in other words, silent) CeD are completely symptom-free even upon detailed questioning. The clinical phenotype has changed over the past 50 years: classical CeD prevailed earlier, whereas non-classical and asymptomatic CeD prevail today[25].

4.3.2 Serology

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 enzyme-linked immunosorbent assay-based measurement of TGA and the immunohistochemistry-based measurement of EMA are the gold standards. In untreated CeD, the sensitivity and specificity of serum TGA-IgA vs duodenal biopsy are both 98%, those of serum EMA-IgA vs duodenal biopsy are 95 and 99%, respectively[19]. In CeD patients with IgA deficiency (1.9% of all cases[26]), IgA-based antibodies can be false negative. Here, TGA-IgG and EMA-IgG are useful markers, although their sensitivity is much humbler compared to their IgA-based counterparts[19]. Since commercial tests use various cut-off values for TGA, measurements >10 times of the upper limit of normal are considered strongly positive.

4.3.3 Endoscopy and histology

Although scalloping, mosaic pattern and fissures are often seen, one-third of patients with a final diagnosis of CeD have a normal duodenal mucosal appearance on upper endoscopy[27]. Duodenal mucosal lesions can be patchy[28] so that at least four

biopsies from the distal duodenum plus at least another sample from the first part should be taken[19,29].

Villous atrophy, which develops as the direct consequence of local Th_1 immune response, 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 (Marsh 4 is no longer in use in clinical practice)[30]. In 1999, Oberhuber et al. fine-tuned this system and divided Marsh 3 into partial (3A), subtotal (3B) and total villous atrophy (3C) based on villous height to crypt depth ratio[31], in which 2:1 is considered normal in the bulb, and 3:1 to 5:1 is considered normal elsewhere in the duodenum[19]. The propagation of intraepithelial lymphocytes—mostly αβ- and γδ-T-cells—is not specific to CeD, >25 intraepithelial lymphocytes/100 epithelial cells are considered abnormal[19].

4.3.4 Genetic testing

HLA-DQ2 or -DQ8 is present in nearly all CeD patients[13] (for details, see subchapter 4.2.2). Since the allele constitution of the DQ heterodimer—particularly the number of copies of HLA DQB1*0201 allele—may be predictive of disease course[17,18], the high-resolution polymerase chain reaction is recommended for HLA-typing. Of note, HLA-typing can be used primarily for ruling-out purposes[19,29] because many people are positive for these haplotypes in the general population, but less than one-tenth of them develop CeD[20].

4.3.5 Diagnostic algorithm

In 1969, the European Society of Paediatric Gastroenterology and Nutrition had proposed the Interlaken criteria which defined CeD based on jejunal histological abnormalities, response to gluten withdrawal and gluten challenge[32]. Since then, serology has revolutionized diagnostics. In the past 50 years, the European Society for Paediatric Gastroenterology, Hepatology and Nutrition released multiple evidence-based guidelines, the last two updates in 2012[33] and 2020[29]. Nevertheless, CeD is no longer known to be a paediatric disease. For diagnosis in adulthood, the American College of Gastroenterology[34] and the European Society for the Study of Coeliac Disease[19] released the most widely used guidelines in 2013 and 2019, respectively. The following paragraphs rely on the recommendations of these guidelines.

In adults, CeD is suspected based on clinical clues (e.g. characteristic signs and symptoms, laboratory abnormalities, high-risk co-morbid conditions, positive family history for CeD). Duodenal histological sampling is mandatory to confirm the diagnosis. The most common scenario is a positive serology followed by endoscopic sampling and histological assessment confirming duodenal villous atrophy (>80% of the cases in our clinic). Seronegative CeD can be diagnosed based on histological evaluation and HLA-typing, while potential CeD can be diagnosed in seropositive and HLA-DQ2 or -DQ8 positive patients without villous atrophy. If the patient is already following a gluten-free diet (GFD), the re-introduction of gluten (i.e. re-challenge) is required before serological testing and histological assessment, both of which can be false negative otherwise. If the patient is negative for HLA-DQ2 and -DQ8, no further diagnostic work-up is necessary to rule out CeD (even during a GFD, the negative predictive value of HLA-typing approaches 100%).

In children, practice tends to implement non-invasive diagnostic strategies (referred to as 'no-biopsy approach') as the duodenal biopsy can be omitted if certain conditions are satisfied (TGA-IgA>10 times of the upper limit of normal with an appropriate test and unequivocally positive EMA from a second serum sample).

As a future perspective, the identification of gluten-reactive T-cells with HLA-DQ-gluten tetramer-based assay is a highly accurate, non-invasive diagnostic tool[35].

4.4 Treatment of coeliac disease: the gluten-free diet

Although having an effective pharmacological treatment is an appealing possibility (for review, see the paper of Singh et al.[36]); today, the only evidence-based treatment option is the complete exclusion of alimentary gluten. The initiation of a lifelong GFD is recommended immediately after the confirmation of the diagnosis of CeD[19,29]. The complete exclusion of dietary gluten results in a vast improvement in gastrointestinal symptoms in the majority of CeD patients within weeks[37].

4.4.1 Definition

As part of a GFD, products containing wheat, barley and rye should be avoided, whereas, proven by a meta-analysis of randomized controlled trials[38], 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)[39]. As per the recommendation of the Codex Alimentarius revised in 2008, a product that is labelled gluten-free should contain <20 ppm gluten (20 mg/kg or

mg/l based on measurement with the enzyme-linked immunosorbent assay R5 Mendez Method)[40].

4.4.2 Dietary adherence

Regular assessment of dietary adherence and dietary education are of utmost importance in CeD. A systematic review identified multiple facilitators and barriers which play a role in maintaining a strict GFD lifelong. These include education and knowledge, health status, motivation, membership in a coeliac association, availability and affordability of gluten-free foods, clear labelling of products, communication with the physician after diagnosis, social awareness and income[41]. Besides, the better the taste, the better the adherence[42]. There is a set of instruments proposed for dietary assessment, though none is perfect (for an excellent review, see the paper of Moreno et al.[43]). The current gold standards for evaluation of dietary adherence include histology, serology and dietary review through interview[19,34].

Repeated duodenal biopsy at 6–24-month on a GFD used to be the gold standard to monitor dietary adherence for decades because mucosal status reflects the inflammatory activity. However, neither a strict GFD[44] nor a clinical response[45] guarantees mucosal recovery. Currently, in adults, a follow-up biopsy is only recommended if symptoms persist despite a strict GFD[19,34].

Serological tests (TGA, EMA, antibodies against deamidated gliadin peptides) are used pervasively to assess dietary adherence. The level of antibodies is expected to decline months after the initiation of a GFD[46], intake of gluten is strongly suspected otherwise[19]. TGA-IgA and EMA-IgA are highly specific (83 and 91%, respectively) but not sensitive indicators (50 and 45%, respectively) of persistent villous atrophy: every second CeD patient with persistent villous atrophy has normal levels of these antibodies[47]. The variability in test performance of the numerous kits at the market limits the comparability of test results[48].

Dietary review through interview executed by a skilled dietician is an effective tool for estimating dietary adherence and, at the same time, for improving patient education. Structured tools, e.g. the Biagi Score[49], the Celiac Dietary Adherence Test[50] or the Gluten-Free Eating Assessment Tool[51], have been proposed to complement (or substitute for) the in-depth interview. However, these suffer from the limitation of reduction of an exhaustive, 10–15-minute, face-to-face interview into a few questions and numbers,

which, more or less, correlate with the results of serological tests and histological assessment. Nevertheless, both the interview and the assessment tools can be biased by relying on patient-reported data.

A promising ancillary instrument is the measurement of GIPs (for further details, see subchapters 4.2.1 and 4.2.3). Peptides resilient to further digestion can be detected from faecal[52] or—as GIPs cross the gut barrier and are excreted by the kidney—from urine samples[53]. Since the amino acid sequences occur in gluten exclusively[9], a positive test is highly suggestive of gluten intake. Urine-GIP detection seems to outperform serological tests in the prediction of intestinal histology at follow-up[53]. Apart from the costs, a drawback of GIP detection is the high individual variability in the clearance and the quick elimination of the peptides (urine-GIPs are detectable for 1–2 days after gluten intake[53]). The routine clinical use of GIP detection is yet to come.

4.4.3 Nutritional implications

At the time of diagnosis, macro- and micronutrient deficiency are common in CeD, tending to improve after the introduction of a GFD. Although a GFD is the only effective therapeutic option, the nutrient profile of gluten-free products falls far from the optimal. As a result, nutritional deficiency, as well as nutritional excess, can develop or persist in some patients following a strict GFD (for an exhaustive systematic review, see the paper of Vici et al.[54] and that of Sue et al.[55]).

Analysing the macronutrient profile of gluten-free products, we can realize poor dietary fibre content[56,57], as fibre derives mainly from the outer layer of the kernel removed and substituted with starch or refined flour during processing. Generally, gluten-free products are rich in carbohydrates with high glycaemic index (e.g. added sugar) and in fat (saturated fatty acids prevail)[56-58]. In line with these, studies reported a higher intake of these nutrients among CeD patients[59,60]. All in all, a 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[61,62], 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[25], 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[62].

4.5 Co-morbid conditions and complications of coeliac disease

Several conditions are the direct consequence of or linked indirectly to CeD; the recognition of these might indicate concomitant screening for CeD[19]. Anaemia, metabolic bone disease and infertility develop on the bases of both malabsorption and chronic inflammation, whereas dermatitis herpetiformis and IgA nephropathy are immune-mediated organ manifestations. Other accompanying pathologies include but are not limited to IgA deficiency, Down's and Turner's syndromes, autoimmune or immune-mediated conditions (e.g. type I diabetes mellitus, autoimmune thyroiditis, Graves' disease, psoriasis), microscopic colitis and ataxia. In the long-term, the increased risk of malignant tumours should be highlighted. In a Dutch study, the crude incidence of enteropathy-associated T-cell lymphoma was 2.08 cases per 100,000 patients >50 years of age[63]. A Swedish study observed a 5.9-fold (CI: 4.3–7.9) increase in the risk of lymphoproliferative malignancies in CeD; in the same time, other malignant tumours, such as colorectal carcinoma and small-bowel adenocarcinoma, were more common as well[64]. The other side of the coin is the slightly reduced risk of breast cancer among women with CeD[64,65]. A feared but seldom (1% lifetime prevalence) complication is refractory CeD (per definition, persistent malabsorption after a 1-year strict GFD), which can be divided to type I (non-malignant, 5-year survival is 80–96%) and type II refractory CeD (pre-malignant, 5-year survival is 43–58%)[66-68]. Besides, acute and chronic cardio-vascular (CV) comorbid conditions have to be listed.

4.6 Mortality and cardiovascular diseases in coeliac disease

In 1999, a study suggested a 3.8 times increase in standardized mortality rate (SMR) among Italian adults with CeD[69]. Many others followed this report[65,70-80], 10 of which established a significantly increased mortality in CeD (Table 1).

The excess mortality mainly results from lymphoproliferative malignancies[80]; however, the role of CV diseases was implicated as well. To date, in CeD, nine studies reported on CV mortality[65,70,71,74-77,79,80], with divergent results (Table 2). A potential cause of this diversity is the β-type error, as four of five studies recruiting a vast number of CeD patients (>10,000) detected a significant increase in CV mortality. In a Swedish study, risk of stroke of any type was significantly higher in CeD (adjusted hazard ratio: 1.10, CI: 1.01–1.19, based on 28,000 cases)[81]. In a UK study, cerebrovascular mortality was higher in CeD patients diagnosed in childhood (SMR: 10.03, CI: 1.21–36)[73]; in contrast, in a Finnish study recruiting patients diagnosed in childhood and adulthood, the risk of

cerebrovascular diseases was lower in CeD (SMR: 0.15, CI: 0.00–0.83)[65]. These studies[65,73] should be interpreted very cautiously due to their limited sample size (340 and 781 cases, respectively). Another small study from Northern Ireland found major adverse cardiac events more frequent in CeD (adjusted hazard ratio: 2.5, CI: 1.22–5.01, based on 349 cases)[82]. A study from the United States of America reported an elevated risk of myocardial infarction (unadjusted odds ratio: 1.73, CI: 1.65–1.82) and coronary artery disease (unadjusted odds ratio: 2.09, CI: 2.03–2.15) in CeD, but—in the absence of controlling for co-variates—these results should be taken as limited evidence[83]. Regarding the risk of venous thromboembolism (VTE), inconsistent findings are available from comparative studies, some of which lack adequate control for covariates (Table 3). Of note, all but one study reporting on the association between CeD and CV diseases investigated cases from European peoples.

Table 1. Adjusted overall mortality in coeliac patients

Studies in chronological order	Country	Age group	N^0 of CeD patients	Relative measure (95% CI)
Cottone et al. 1999[69]	Italy	Adults	216	**SMR: 3.8 (2.0–7.0)**
Corrao et al. 2001[70]	Italy	Adults	1,072	**SMR: 2.0 (1.5–2.7)**
Peters et al. 2003[71]	Sweden	Mixed	10,032	**SMR: 2.0 (1.8–2.1)**
West et al. 2004[72]	UK	Mixed	4,732	**HR: 1.3 (1.0–1.6)**
Viljamaa et al. 2006[65]	Finland	Mixed	781	**SMR: 1.3 (1.0–1.6)**
Solaymani-Dodaran et al. 2007[73]	UK	Adults	340	**SMR: 1.6 (1.3–1.8)**
Ludvigsson et al. 2009[74]	Sweden	Mixed	29,096	**HR: 1.4 (1.3–1.5)**
Grainge et al. 2011[75]	UK	Mixed	1,092	**SMR: 1.4 (1.2–1.6)**
Abdul Sultan et al. 2015[76]	UK	Mixed	10,825	HR: 0.9 (0.8–1.0)
Holmes and Muirhead 2018[77]	UK	Adults	2,174	**SMR: 1.6 (1.4–1.8)**
Quarpong et al. 2019[78]	Scotland	Mixed	602	**SMR: 2.1 (1.4–3.0)**
Lebwohl et al. 2020[79]	Sweden	Mixed	49,829	**HR: 1.2 (1.2–1.3)**
Koskinen et al. 2020[80]	Finland	Adults	12,803	HR: 1.0 (0.9–1.1)

Regarding relative measures, boldface type indicates a statistically significant association. All studies controlled for potential confounding factors. CeD: coeliac disease; CI: confidence interval; HR: hazard ratio; SMR: standardized mortality rate.

Table 2. Adjusted cardiovascular mortality in coeliac disease

Studies in chronological order	Country	Age group	N⁰ of CeD patients	Relative measure (95% CI)
Corrao et al. 2001[70]	Italy	Adults	1,072	SMR: 0.7 (0.3–1.5)
Peters et al. 2003[71]	Sweden	Mixed	10,032	**SMR: 1.6 (1.4–1.8)**
Viljamaa et al. 2006[65]	Finland	Mixed	781	SMR: 1.2 (0.83–1.68)
Ludvigsson et al. 2009[74]	Sweden	Mixed	29,096	**HR: 1.19 (1.11–1.28)**
Grainge et al. 2011[75]	UK	Mixed	1,092	SMR: 1.12 (0.82–1.50)
Abdul Sultan et al. 2015[76]	UK	Mixed	10,825	**CIF: –1.4% (–1.9 to –0.9%)**
Holmes and Muirhead 2018[77]	UK	Adults	2,174	SMR: 1.23 (0.98–1.51)
Lebwohl et al. 2020[79]	Sweden	Mixed	49,829	**HR: 1.08 (1.02–1.13)**
Koskinen et al. 2020[80]	Finland	Adults	12,803	HR: 0.91 (0.77–1.07)

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

Table 3. Risk of venous thromboembolism in coeliac disease

Studies in chronological order	Country	N⁰ of CeD patients	Type	Relative measure (95% CI)
Miehsler et al. 2004[84]	Austria	207	VTE	RR: 0.4 (0.06–2.50)
Ludvigsson et al. 2007[85]	Sweden	14,207	VTE	**HR: 1.86 (1.54–2.24)**
Ramagopalan et al. 2011[86]	UK	1,722	VTE	RR: 1.35 (0.89–1.97)
		2,324		RR: 1.36 (0.87–2.03)
		48,239		**RR: 1.21 (1.11–1.33)**
Zöller et al. 2012[87]	Sweden	13,940	PE	**SMR: 1.53 (1.30–1.78)**
Johannesdottir et al. 2012[88]	Denmark	136,176	VTE	OR: 1.0 (0.8–1.4)
			DVT	OR: 0.9 (0.6–1.3)
			PE	OR: 1.4 (0.9–2.3)

Regarding relative measures, boldface type indicates a statistically significant association. CeD: coeliac disease; CI: confidence interval; DVT: deep vein thrombosis; HR: hazard ratio; OR: odds ratio; PE: pulmonary embolism; RR: risk rate; SMR: standardized mortality rate; VTE: venous thromboembolism.

4.7 Prothrombotic alterations in coeliac disease

Virchow had proposed that main events needed for thrombus formation include endothelial injury, hypercoagulability and blood flow abnormalities[89]. In CeD, theoretically, the fragile balance of pro- and antithrombotic factors can be disturbed in many ways (Fig 5).

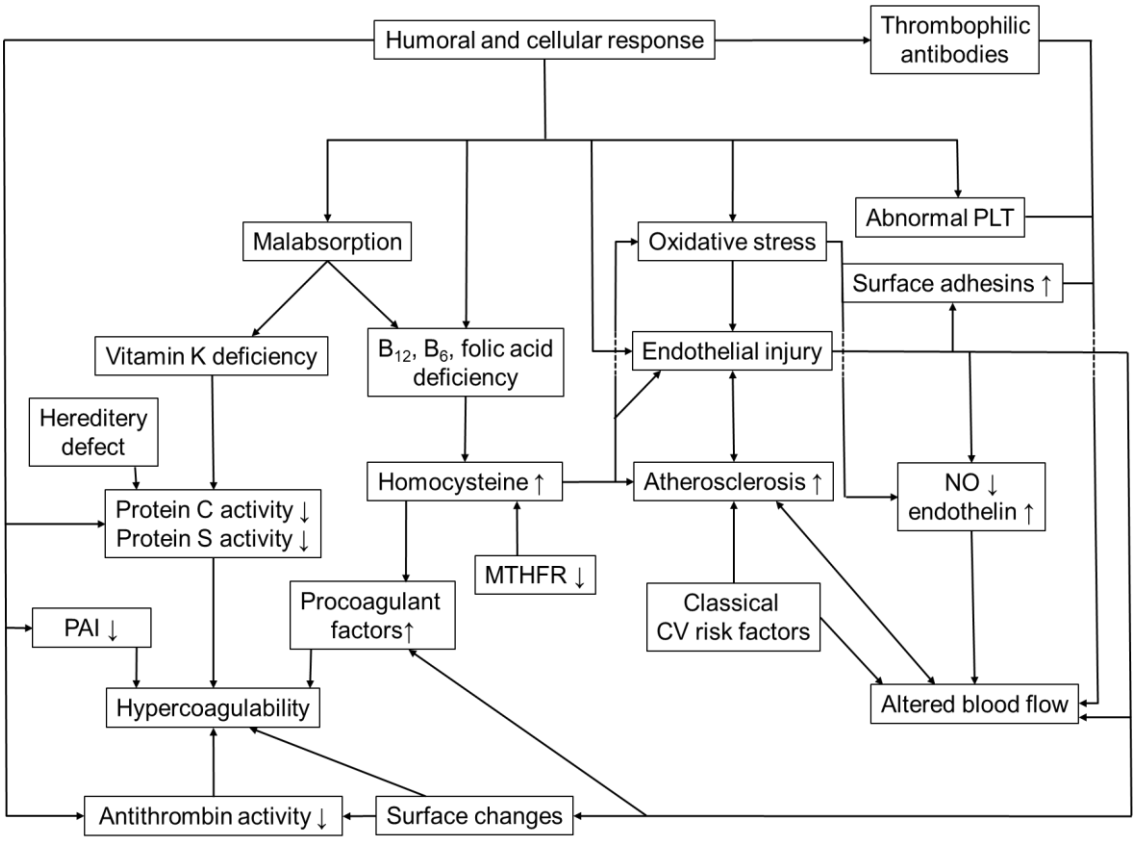


Fig 5. Model for the interplay of pro-thrombotic alterations in coeliac disease. CV: cardiovascular; MTHFR: methylene tetrahydrofolate reductase; NO: nitrogen oxide; PAI: plasminogen activator inhibitor; PLT: platelet. The figure is the author's own work.

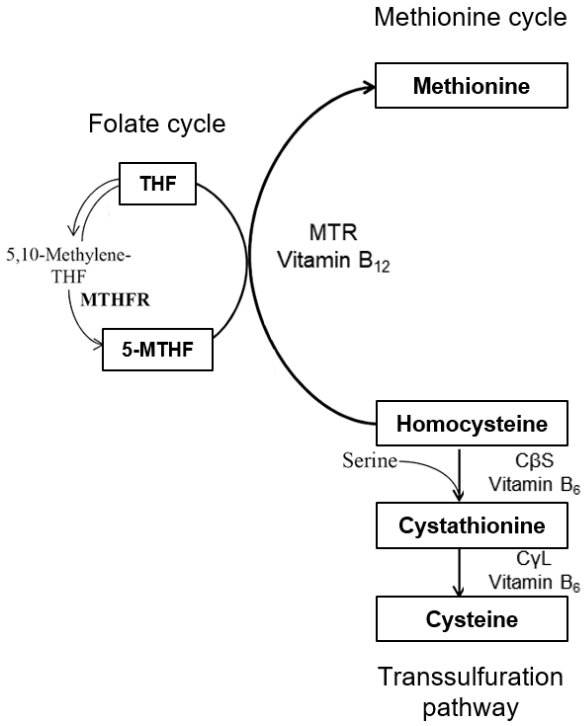


Fig 6. Metabolism of homocysteine. Hyperhomocysteinaemia occurs if homocysteine is not converted to cysteine in the B_6-dependent transsulfuration pathway or to methionine in the B_{12}- and folic acid-dependent methionine-synthase reaction. CβS: cystathionine beta synthase; CγL: cystathionine gamma-lyase; MTHF: methylene tetrahydrofolate; MTHFR: methylene tetrahydrofolate reductase; MTR: methionine synthase reductase; THF: tetrahydrofolate. Reprinted and adapted from 'Three Main Causes of Homocystinuria: CBS, cblC and MTHFR Deficiency. What do they Have in Common?' by Hoss et al., 2019, *J Inborn Errors Metab Screen*.

Resulting from malabsorption, vitamin B_{12}, folic acid and vitamin B_6 deficiency lead to hyper-homocysteinaemia[90,91] (Fig 6[92]). Loss-of-function mutations of methylene tetrahydrofolate reductase enzyme were proposed to contribute to the elevated homocysteine level in CeD[93]; however, a following controlled study did not confirm the accumulation of mutant alleles in CeD patients compared to the control group[94]. Hyperhomocysteinaemia (>15 μmol/L) has a variety of untoward effects including but not restricted to endothelial damage, oxidative stress, impaired nitrogen oxide production, increased activity of pro-coagulant and suppressed activity of anti-coagulant coagulation factors, lipid peroxidation and low-density lipoprotein (LDL) oxidation (for review, see the paper of Djuric et al.[95]).

Natural anticoagulants, such as protein C, protein S and antithrombin, serve as physiological inhibitors of the coagulation cascade. Protein C, activated by thrombin, and protein S, the co-factor of protein C, provide a negative feedback mechanism to limit coagulation (Fig 7)[96]. These factors are produced in the liver in vitamin K-dependent reactions. As a result of fat malabsorption, vitamin K deficiency is expected to develop in CeD. Protein C and protein S deficiency were identified when investigating the aetiology of thrombotic events in CeD[97-99]. Antithrombin, a glycoprotein, is a serine pro-tease inhibitor in the feedback system of the coagulation cascade, neutralizing thrombin, factors IXa, Xa, XIa and XIIa. Although antithrombin is produced in the liver

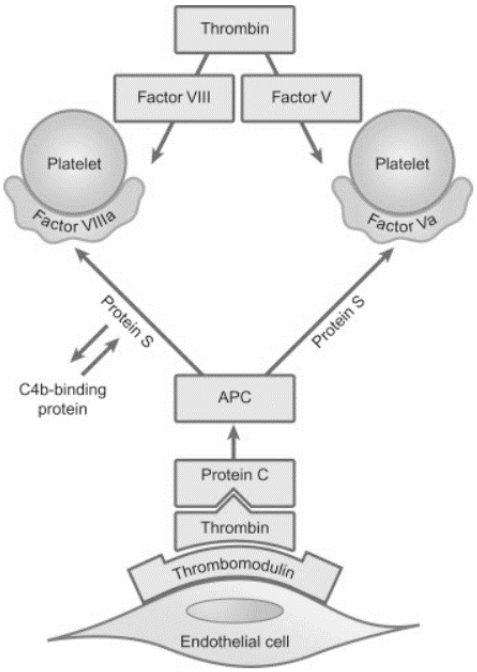


Fig 7. Mechanism of action of natural anticoagulants. Activated protein C is the co-factor of protein S, which inhibits factors Va and VIIIa. APC: activated protein C; C4b: complement-4b. Reprinted from 'Hypercoagulability: a new cofactor in the protein C anticoagulant pathway' by Bauer et al., 1994, *N Eng J Med*.

independently of vitamin K, malnutrition and inflammation can reduce its activity[100]. Despite our efforts, we could not identify any study that measured the activity of antithrombin in CeD.

Oxidative stress is substantial in CeD and correlates with the severity of intestinal mucosal damage[101].

Endothelial injury in CeD is of multifactorial aetiology, in which the interplay of hyperhomocysteinaemia, increased oxidative stress, subclinical chronic inflammation and nutritional causes is implicated[102]. The dysfunctional endothelium is no longer protective against thrombus formation; in fact, it promotes the adhesion of circulating cellular elements and thrombus formation, mainly via the regulation of vascular tone by nitrogen oxide and endothelin[95,103,104]. Altered serotonin metabolism in platelets can promote cellular activation and adhesion as well[105].

Not only the endothelial injury but also the inflammation activates the coagulation cascade via several mechanisms, such as by the inhibition of plasminogen activator inhibitor-1, antithrombin, tissue factor pathway inhibitor, by the release of tissue factor and by the activation of platelets (for review, see the paper of Aksu et al.[106]). Thrombophilic antibodies—mainly antiphospholipid antibodies, e.g. cardiolipin IgG and IgM, prothrombin IgG, anti-β2 glycoprotein I, antiphosphatidylserine-prothrombin IgM and IgG, but an interplay between TGA and factor XIII is implicated as well[107]—are potential contributors of hypercoagulability in CeD[108]. Besides, autoimmune diseases, being associated with hypercoagulability (e.g. antiphospholipid syndrome, systemic lupus erythematosus or inflammatory bowel diseases), often co-occur with CeD[109].

Atherosclerosis accelerates in CeD, which is well reflected by the increased carotid intima-media thickness[104,110]. Plaque formation is promoted by subclinical chronic inflammation (active CD8+ T-cells and circulating interferon-γ)[111] and endothelial

injury[112]. Atherosclerotic plaques, vasoactive mediators and endothelial injury can change blood flow: either stasis or turbulent flow can occur. To our best knowledge, properties of blood flow have never been investigated in CeD.

We have little information whether prothrombotic alterations persist or disappear during a GFD. If malabsorption recovers, the level of vitamin K[113], thereby the activity of protein C and protein S are expected to restore. On the contrary, reports indicated that hyperhomocysteinaemia[90,104,114] and thrombophilic antibodies[108] could persist in the long-term. Chronic inflammation can also persist, as indicated by mucosal damage despite being gluten-free for years[44]. Data on the preventive effect of a GFD on autoimmune co-morbid conditions are seldom and very limited. Obesity and non-alcoholic fatty liver—singly or as part of the metabolic syndrome—can develop due to the suboptimal nutrient profile of a GFD (for details, see subchapter 4.4.3). Taken together, we have no solid evidence if hypercoagulability, endothelial injury and impaired blood flow persist during a GFD.

4.8 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[115]. Within the scope of rheology, haemorheology deals with the rheological properties of blood and its cellular elements. The following sections rely on the content of the review papers of Baskurt and Meiselman (2003)[115], Késmárky et al. (2008)[116] and Pretorius (2018)[117].

4.8.1 Blood from a rheological point of view

In medicine, blood is a liquid tissue comprising formed elements (that is, red blood cells [RBC], white blood cells and platelets) and intercellular material (that is, plasma). In rheology, blood is a two-phase liquid or a solid-liquid suspension, where the formed elements serve as solid components. At a given temperature and shear rate, the fluidity of the blood is determined by the rheological properties of plasma and the cellular phase, and by the fraction of the cellular phase (i.e. haematocrit). An essential term in rheology is viscosity, defined as the intrinsic property of fluids that indicates resistance to flow (by the equation, shear stress divided by shear rate). Whole blood viscosity (WBV) is the most important in vivo rheological parameter.

4.8.2 Viscosity

Blood is a non-Newtonian fluid, meaning that its viscosity is not constant across varying shear stresses. Blood shows a shear-thinning behaviour: the apparent viscosity is relatively high at low shear stresses, whereas it becomes low at high shear stresses. WBV is always higher than plasma viscosity (PV) because plasma does not contain the cellular elements.

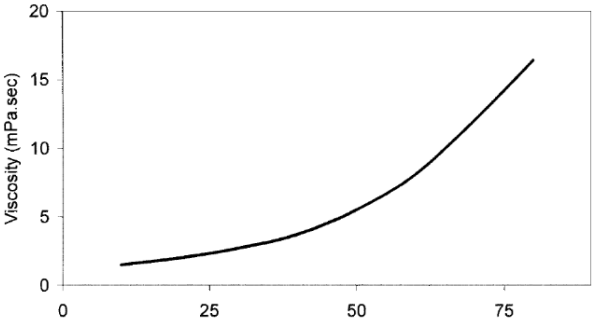


Fig 8. Association between whole blood viscosity and haematocrit. The higher the haematocrit, the higher the viscosity. Reprinted from 'Blood rheology and haemodynamics' by Baskurt and Meiselman, 2003, *Semin Thromb Haemost*.

A unit change in haematocrit—the primary determinant of WBV (Fig 8)—increases WBV by 4% at medium to high shear rates. In haematocrit, both relative and absolute elevation (haemoconcentration and polyglobulia, respectively) matter. Besides, an increase in PV elevates WBV as well.

Plasma is a Newtonian fluid, meaning that PV is independent of the shear stress. At 37°C, PV falls between 1.10–1.35 mPa·sec (for reference, the viscosity of water is 1.00 mPa·sec), which can rise to 5–6 mPa·sec under pathological circumstances. PV is mainly determined by the macromolecule content of the plasma, of which fibrinogen (an acute-phase protein) is the primary determinant (others include albumin, immunoglobulins and paraproteins). PV increases in many conditions, such as acute and chronic inflammation and myeloproliferative neoplasms.

4.8.3 Erythrocytes

RBCs, the most abundant cells in the blood (the normal range is 4.5–5.5 T/L), 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; on average, 8 μm in diameter and 2 μm in thickness) 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. The

properties of the membrane can be altered in hereditary (e.g. spherocytosis) and acquired conditions (e.g. liver cirrhosis, uraemia), and are strongly determined by protein and lipid (e.g. cholesterol, phospholipid) content. In addition to structural factors, energy-dependent processes, e.g. the transmembrane osmotic gradient and the intracellular Ca^{2+} concentration, affect mechanical properties as well. Intracytoplasmic viscosity is determined by haemoglobin, of which both the quality and the quantity can be altered.

Fig 9. Rouleaux formation. Reprinted from 'Blood rheology and haemodynamics' by Baskurt and Meiselman, 2003, *Semin Thromb Haemost*.

RBCs tend to arrange like a stack of coins, termed as rouleaux, in autologous plasma at rest (Fig 9). Aggregates readily dissolve to forces. Two, not mutually exclusive models have been proposed to explain the phenomenon of EA. The "Bridging Model" says that aggregating forces derived from macromolecules in between the nearby RBCs overcome disaggregating forces. According to the "Depletion Model", the repellent effect of surface macromolecules to plasma macromolecules causes an osmotic gradient and consequent water current from the intercellular gaps, resulting in a drop in cell-solvent affinity. In both theories, macromolecules of the plasma and that of RBCs' surface are involved. Since macromolecules are essential regarding PV, determinants of EA partly overlap that of PV. Fibrinogen plays a critical role; other macromolecules promoting RBC-RBC interactions also affect EA.

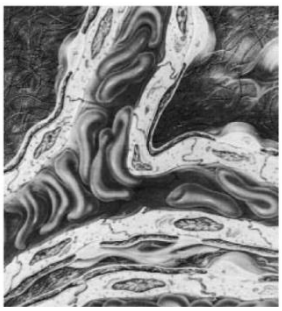


Fig 10. Erythrocyte deformability in the microcirculation. Reprinted from 'Blood rheology and haemodynamics' by Baskurt and Meiselman, 2003, *Semin Thromb Haemost.*

Both ED and EA are essential in determining WBV. 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 (Fig 10); besides, aggregates can plug capillaries so that resistance to flow increases.

4.8.4 White blood cells and platelets

Under physiological circumstances, white blood cells and platelets account for the minority of haematocrit and, therefore, play a little part in determining WBV. They can cause hyperviscosity in leukocytosis or thrombocytosis.

4.8.5 Haemorheological alterations in immune-mediated disorders

Haemorheological alterations are present in many immune-mediated disorders including systemic lupus erythematosus[118], rheumatoid arthritis[118,119], Raynaud syndrome[120] and systemic sclerosis[121].

In gastroenterology, studies extensively investigated haemorheology in inflammatory bowel disease and reported prominent—sometimes, activity-dependent—prothrombotic alterations of fibrinogen, PV, ED and EA[122-127]. ED is impaired in both active and inactive inflammatory bowel disease compared to a control group[122], and the strength of aggregation increases significantly with the disease compared to a control group[123]. In two studies[124,125], Crohn's disease, compared to a control group, was accompanied with a lower level of haematocrit, a higher level of fibrinogen, and increased EA and PV. The level of fibrinogen, EA[125] and PV[126] are increased in active disease compared to inactive disease, and the level of fibrinogen[124] and PV[126] significantly correlate with the Crohn's Disease Activity Index. In ulcerative colitis, the level of fibrinogen, EA and PV increase with the disease compared to a control group as well as with active disease compared to inactive disease[125,127]. PV correlates with clinical, endoscopic and histological severity scores of ulcerative colitis[127].

To our knowledge, no studies investigated haemorheological parameters in CeD.

5. 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 5). 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.

5.1 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.

5.2 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 prothrombotic haemorheological alterations and altered 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.

6. Methods

Reporting the study, we adhered to the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) Statement (2007)[128]. The study investigated patients with CeD or inflammatory bowel disease compared to control subjects, but here, I report only CeD-related parts as per the objectives of the thesis. (Findings on inflammatory bowel disease will be published later.)

6.1 Ethics and pre-study protocol

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 at https://www.isrctn.com/) and published it in the BMJ Open[129] before starting the recruitment.

6.2 Study design, setting

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. Recruitment did not change regular care.

My supervisor, dr Judit Bajor, was the Principal Investigator of the study; and I was the Trial Coordinator. For the roles and duties, see the pre-study protocol.

6.3 Eligibility criteria

Inclusion criteria for **all participants**:

- Age had to be ≥18 years.
- Blood collection had to be indicated independently of the study.
- Signed informed consent had to be provided.

Inclusion criteria specifically for **CeD patients**:

- The newly diagnosed or followed cases, regardless the age and date of diagnosis, had to be histology-confirmed; otherwise, the diagnostic criteria of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (2012) for confirming CeD in children[33] or those of the American

College of Gastroenterology (2013) for confirming CeD in adults[34] had to be fulfilled.

Inclusion criteria specifically for **control subjects**:

- CeD had to be excluded based on the recommendation of the guidelines[33,34]. Subjects had to be on a gluten-containing diet.
- Inflammatory bowel disease had to be excluded based on the recommendation of the European Crohn's and Colitis Organisation (available online at https://www.ecco-ibd.eu/).

Exclusion criteria for **all participants**:

- advanced chronic conditions:
  - liver cirrhosis (Child–Pugh B–C);
  - chronic kidney disease—stage 3 or more severe based on the estimated glomerular filtration rate calculated with the Chronic Kidney Disease Epidemiology Collaboration;
  - chronic heart failure (New York Heart Association III–IV);
  - any active malignant disease;
- any acute diseases or invasive procedures within 2 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) or antiplatelet drugs;
- confirmed systemic lupus erythematosus;
- pregnancy.

6.4 Flow and timing

After obtaining informed consent, we, assisted by clinical research administrators, collected clinical data and biological samples by schedule shown in Table 4. During data collection, we assessed baseline characteristics (including complete past medical history and medications going back to the past 3 months) by detailed questioning complemented with the revision of electronic and printed medical files. Then, we completed the thrombophilia and symptom score questionnaires. The visit ended with a dietary interview, followed by urine and blood collection in the central laboratory unit of the university.

Table 4. Schedule for the study

	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			
Assessment					
Baseline characteristics			x		
Thrombophilia questionnaire			x		
Symptom score			x		
Dietary adherence			x		
Urine collection				x	
Blood collection				x	
Blood analysis					x
Urine analysis					=>*

*samples were deep-frozen until all participants have been recruited.

6.5 Questionnaires and measurements

Items of the thrombophilia questionnaire were selected based on two review papers[130,131] and were chosen to cover both arterial and venous risk factors of thrombus formation, as follows:

- sex, age;
- history of arterial and venous thrombotic events, hereditary thrombophilias;
- family history of arterial and venous thrombotic events, hereditary thrombophilias (among first-degree relatives);
- current medications (with particular emphasis on anticoagulants and antiplatelet agents);
- gynaecological history (with particular emphasis on pregnancy, pregnancy complications and abortions, hormone replacement therapy, oral contraceptives)
- smoking, alcohol and drug abuse;
- comorbidities (with particular emphasis on malignant tumours, hypertension, diabetes mellitus, obesity; CV, respiratory and kidney diseases; peripheral occlusive arterial disease, lipid metabolism disorders and immune-mediated disorders) and surgical history;
- immobilization (bedrest >3 days), trauma, plaster cast in the past 3 months;
- long travels (>6 hours) by car, plane or bus in a continuous sitting position;

- lower limb varicose veins or chronic venous insufficiency;
- acute infection in the past 2 weeks (with particular emphasis on common respiratory, urinary or gastrointestinal symptoms);
- invasive diagnostic or therapeutic intervention in the past 2 weeks.

The severity of symptoms was rated employing the Gastrointestinal Symptoms Rating Scale, a 15-item tool assessing each item on a Likert scale between 1 and 7[132].

Laboratory measurements were performed after overnight fasting from venous blood (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 seven plastic tubes and 42.7 ml blood from each patient [BD, USA]) by the Department of Laboratory Medicine, by the Department of Immunology and Biotechnology and by the Haemorheological Laboratory, First Department of Medicine (Medical School, University of Pécs). Measurements included

- routine laboratory parameters: urea, creatinine, cholesterol (total, high-density [HDL] and low-density lipoproteins), triglyceride, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transferase, bilirubin, total protein, albumin, immunoglobulins, C-reactive protein, vitamin B_{12}, blood counts, and erythrocyte sedimentation rate;
- immunological indicators: CeD-specific antibodies (TGA IgA/G, EMA IgA) and antiphospholipid antibodies (lupus anticoagulant, cardiolipin IgG/A/M, B2-glycoprotein-I IgG/A/M, prothrombin IgG/A/M);
- haemostatic parameters: prothrombin, thrombin time, activated partial thromboplastin time, fibrinogen; the activity of antithrombin, protein C, and protein S;
- homocysteine;
- haemorheological parameters (for related terms, see Table 5):
  - fibrinogen;
  - haematocrit measured with the microcapillary method;
  - EA (Fig 11[133]), described with the aggregation index (AI, given in %), aggregation half-time ($t_{1/2,}$ given in sec) and threshold shear rate (γ, given in 1/sec), measured with the Laser-assisted Optical Rotational Cell Analyzer (LORCA, R&R Mechatronics, Hoorn, The Netherlands);
  - ED, described with the elongation index (EI, no unit) measured at nine shear stresses between 0.3–30 Pa—low and high shear stresses fall between 0.3–

1.69 and 3–30 Pa, respectively—with laser-diffraction ektacytometry with a LORCA (elongation of RBCs is illustrated in subchapter 7.2.1 by Fig 13 inlet);

- WBV and PV measured with the Brookfield DV-III Ultra LV Programmable rotational viscometer (Brookfield Engineering Labs; Middleboro, Massachusetts, USA) at mid-shear (90 1/sec).

Heamorheological measurements were performed within 2 hours after sampling in line with the recommendations of the International Expert Panel for Standardization of Haemorheological Methods[134].

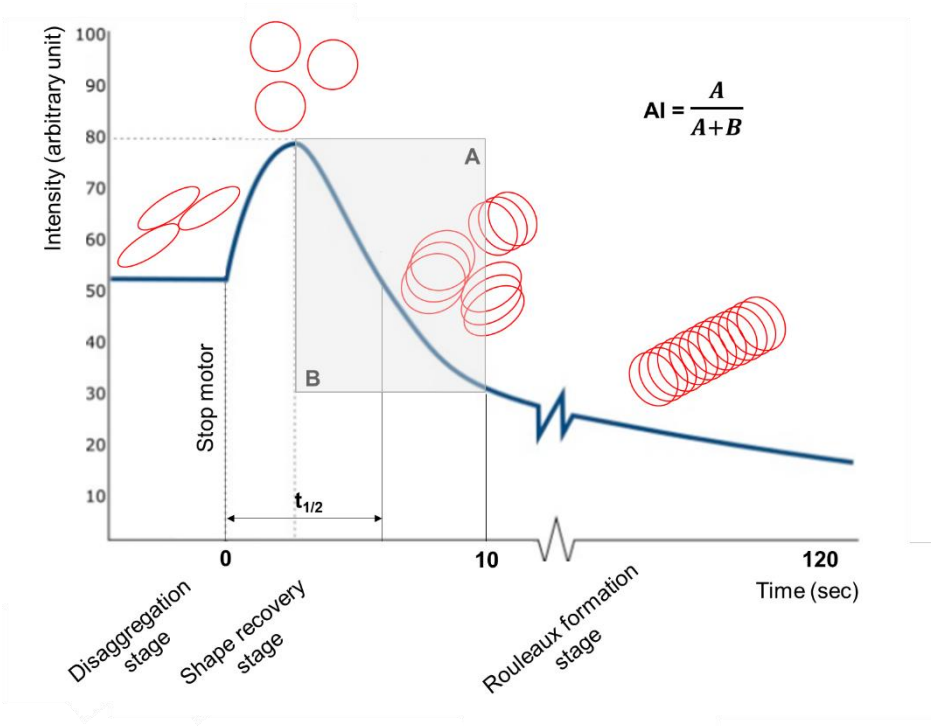


Fig 11. Syllectogram of erythrocyte aggregation. The intensity of laser block scatter (vertical axis) is plotted against time (horizontal axis). After complete disaggregation, erythrocytes (depicted with the red circles and ellipses in the figure) regain their shape (causing an upstroke in light intensity, right after 0 sec in the figure) and start aggregating. The aggregation index is calculated based on the equation given in the right upper quadrant of the figure, where A and B represent areas of the grey square. $t_{1/2}$: aggregation half-time; AI: aggregation index. Reprinted and adapted from 'Changes in Red Blood Cell Properties and Platelet Function during Extracorporeal Membrane Oxygenation' by Lansink-Hartgring et al., 2020, *J Clin Med*.

Table 5. 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 and low shear stresses (shown in Fig 13 inlet)	↓
Erythrocyte aggregation	Aggregation index (%)	integral in the change of light intensity 10 sec after disaggregation (shown in Fig 11)	↑
	Aggregation half-time (sec, symbol: $t_{1/2}$)	the time required for achieving half of the maximal aggregation after disaggregation (shown in Fig 11)	↓
	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)	↑
	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.

Mid-stream urine, approximately 100 ml from each participant, was collected in a sterile plastic container and stored at –80 °C until processing. We measured urine-GIP with a point-of-care test (iVYCHECK GIP Urine, Biomedal, Spain), an immuno-chromatographic dipstick containing G12 monoclonal antibodies targeted against 33-mer GIP. If peptide concentration reaches the detection threshold, a line appears near the control line within 30 minutes after dipping (for further technical information, see the manufacturer's website at https://ivydal.biomedal.com/).

6.6 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 6 summarizes the characteristics of the modalities used for the estimation of dietary adherence, for further information, see subchapters 4.4.2 and 6.5.

Table 6. Comparative description of tools used for the estimation of dietary adherence in coeliac disease

	Coverage	Assessment	Resources		Invasiveness	Time to result	Availability in Hungary	Correlation	
			Human resources	Disposals				Mucosal damage	Symptoms
Urine-GIP detection (with a point-of-care dipstick)	days	objective, semi-quantitative[a]	nurse or technician[b]	urine container plus 4,000 HUF/dipstick	non-invasive	same-visit	for research purposes	good	moderate
Dietary review through interview	weeks to months	subjective, quantitative or qualitative[c]	skilled dietician[d]	none	non-invasive	same-visit	wide or limited	variable[c]	variable[c]
CeD-specific antibodies[e]	3–6 months	objective, quantitative	nurse and technician[f]	plastic tube plus 5,000 HUF/test	blood collection	days	wide	poor	moderate

[a]depending on test type and equipment, [b]buffering, dipping, then reading 30 minutes later, [c]validated standards for assessment are not available, [d]a thorough interview takes 10-20 minutes to be carried out and requires years of work experience, [e]EMA-IgA, TGA-IgA, -IgG, [f]nurse for blood collection, a technician for sample preparation and processing.
CeD: coeliac disease; GIP: gluten immunogenic peptide; HUF: Hungarian Forint.

6.7 Outcomes and blinding

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.

Personnel involved in the administration of the questionnaires and the execution of the dietary interview were not aware of laboratory results because of the chronological order of the procedures (Table 4) but were aware of group allocation (CeD vs control). Laboratory personnel were blind to group allocation and all data collected within the study.

6.8 Data management

Participants were given a three-digit subject identifier, used subsequently during the entire study, immediately after giving informed consent. Data were collected onto de-identified, paper-based case report forms stored in locked cabinets. On completion of data collection, clinical research administrators transferred the data onto the corresponding columns of a de-identified master sheet. First, authorized clinical research administrators and I checked the data quality; then, after revision, the Principal Investigator closed the cases. Having closed all the cases, we forwarded the master sheet and the hypotheses of the study to statistical analysis.

6.9 Sample size and statistical analysis

We planned the study to be a two-phase study. Since haemorheological parameters were never investigated in CeD, 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 (>1,000 subjects for parameters describing EA, 247 subjects for PV and 289 subjects for WBV per group), so that we decided to stop the study for lack of feasibility.

In analysis, after matching by age (±5 years tolerance) and sex (±10% tolerance) in 1:1 ratio, descriptive statistics were performed. Categorical variables were given in proportions (% of total). Continuous variables were given with central tendencies (mean or median) and measure of dispersion (standard deviation, quartiles or range) based on distribution determined by the visual inspection of Q–Q plots. Logarithmic transformation

was applied to normalise the distribution in the case of $t_{1/2}$, γ, WBV, PV, and the activity of antithrombin and protein S (the figures were created based on non-transformed data).

In univariate analysis, we used the Welch, Mann-Whitney, Chi^2- and Fisher's tests when comparing CeD patients to control subjects (α=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 (those above the line at zero). During prediction, 100 random forests with 500 trees in each were grown using conditional inference method to avoid bias toward dependent predictors and overfitting. Models were created for the whole study population with 34 variables to determine whether CeD is an independent predictor of the outcome. Additional models, applicable to CeD patients only, were created by adding CeD-specific variables (that is, dietary adherence, length of a GFD, symptom score, age at diagnosis of CeD) to the initial set of variables to determine if these predict the outcomes independently.

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) by statistician Nelli Farkas.

7. Results

7.1 Characteristics of the participants included

We screened a total of 162 subjects for eligibility, 126 of which proved to be eligible for inclusion in the study. After matching, data of 50 CeD patients and 50 control subjects were analysed (for flowchart, see Fig 12).

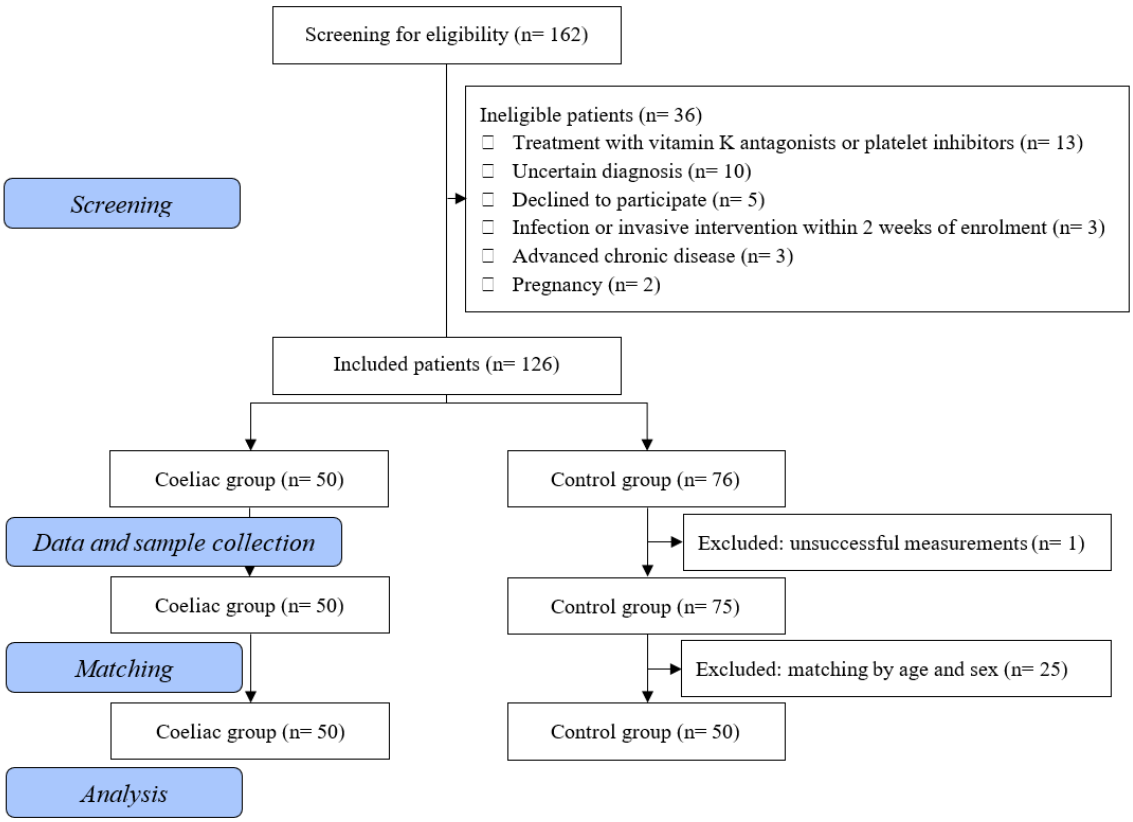


Fig 12. Flowchart of the study. The figure is the author's own work.

We did not find any significant difference in age, sex and other clinical characteristics between CeD patients and matched control subjects (Table 7). 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 8). One CeD patient and two control subjects received statin therapy for known hypercholesterinaemia; regular medications and comorbidities of participants are listed in Appendix 1.

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). At the time of the study, 6 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. CeD patients rated a median of 1.50 points on the Gastrointestinal Symptoms Rating Scale (p=0.790 for the comparison with the values of control subjects, see Appendix 2).

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 7. 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)
Chronic alcohol abuse (n, %)	3 (6.0)	2 (4.0)
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)
Lower limb varicose veins (n, %)	14 (28.0)	11 (22.0)
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 8. 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*
Creatinine (μmol/L)	71.6; 68.5 [39.0–110.0]	72.8; 68.5 [49.0–105.0]	0.668
Carbamide (mmol/L)	4.2; 4.1 [2.2–7.3]	4.5; 4.3 [2.2–7.9]	0.257
Total bilirubin (umol/L)	8.1; 7.9 [2.7–20.0]	9.5; 8.1 [2.1–24.5]	0.120*
AST (U/L)	25.6; 19.0 [10.0–200.0]	22.0; 20.0 [10.0–89.0]	0.806*
ALT (U/L)	24.1; 18.0 [9.0–158.0]	21.7; 18.0 [8.0–60.0]	0.901*
ALP (U/L)	74.3; 68.0 [36.0–269.0]	67.9; 67.5 [35.0–108.0]	0.696*
γ-GT (U/L)	23.1; 16.0 [10.0–94.0]	26.1; 16.0 [7.0–210.0]	0.825*
Total protein (g/L)[a]	74.5; 74.7 [62.4–93.0]	75.3; 74.7 [67.7–85.2]	0.445
Albumin (g/L)	48.2; 47.8 [37.8–56.6]	49.2; 49.1 [43.9–57.8]	0.182
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 (%)	59; 59 [41–83]	58; 58 [43–74]	0.353
Neutrophil granulocytes (G/L)	4.4; 4.2 [1.7–13.4]	3.9; 3.6 [1.8–9.2]	0.134
Lymphocytes (%)	30; 29 [9.0–45.3]	32; 33 [16–47]	0.171
Lymphocytes (G/L)	2.1; 2.0 [1.2–4.2]	2.1; 2.1 [1.1–3.9]	0.947
Monocytes (%)	7.3; 7.0 [2.6–11.5]	7.4; 7.4 [4.2–10.7]	0.773
Monocytes (G/L)	0.52; 0.48 [0.15–1.14]	0.50; 0.47 [0.18–0.99]	0.469
Eosinophil granulocytes (%)	2.5; 2.0 [0.3–8.5]	1.8; 1.5 [0.0–7.8]	**0.023**
Eosinophil granulocytes (G/L)	0.18; 0.14 [0.01–0.60]	0.11; 0.10 [0.00–0.47]	**0.003**

Table 8 (continued)

	Coeliac group (n=50)	Control group (n=50)	p-value
Basophil granulocytes (%)	0.77; 0.60 [0.30–4.00]	0.70; 0.70 [0.20–1.90]	0.487
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. γ-GT: γ-glutamyl transferase; ALP: alkaline phosphatase; ALT: alanine aminotransferase; APTI: activated partial thromboplastin time; AST: aspartate aminotransferase; 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.

7.2 Coeliac patients vs control subjects

7.2.1 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 13); implying an impaired ED in CeD. Random forest analysis confirmed that CeD is an important predictor of EI at high shear stresses (Fig 14) but not at low shear stresses (Appendix 3).

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 9). In line with these, random forest analysis did not highlight CeD as an important predictor of any of these outcomes (Appendix 3).

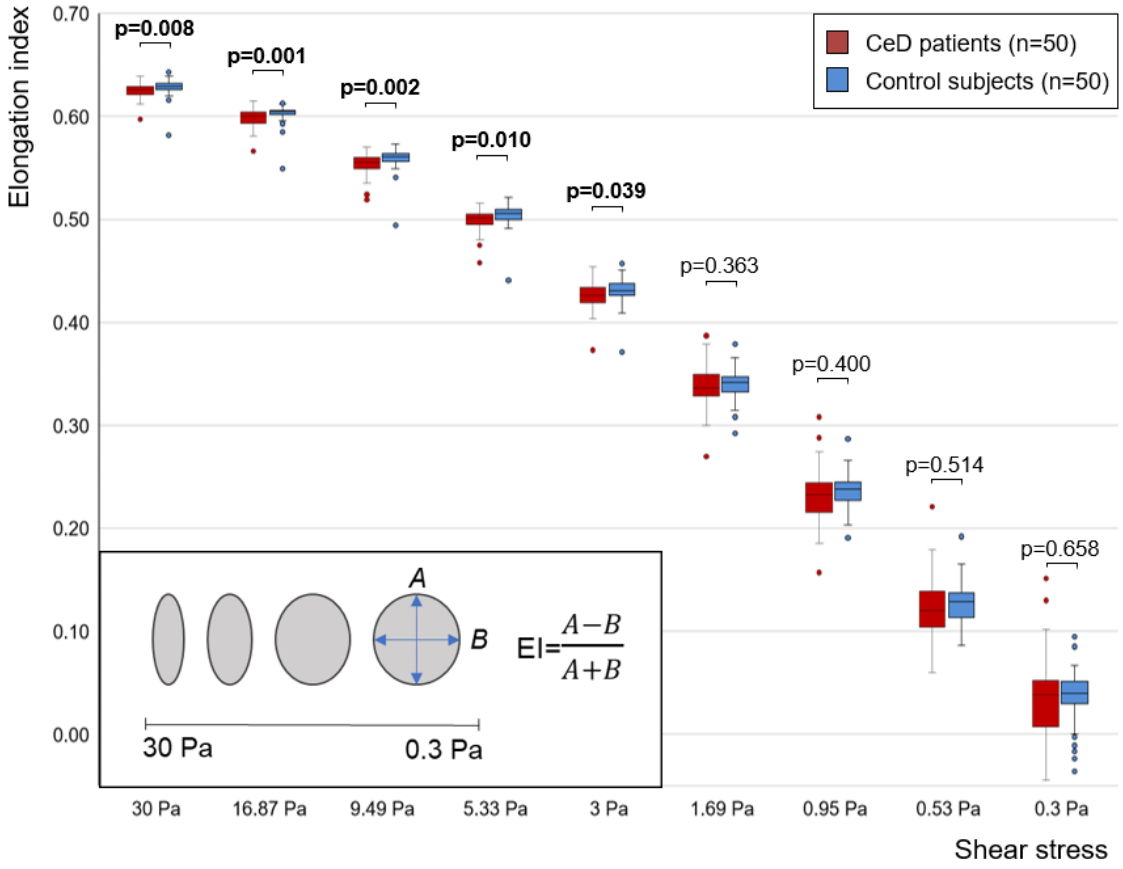


Fig 13. Erythrocyte deformability at different shear stresses (ektacytogram) in coeliac patients vs control subjects. The horizontal axis shows different shear stresses from 0.3 to 30 Pa; the vertical axis shows the elongation index. P-values were generated with the Mann-Whitney test, boldface type indicates 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.

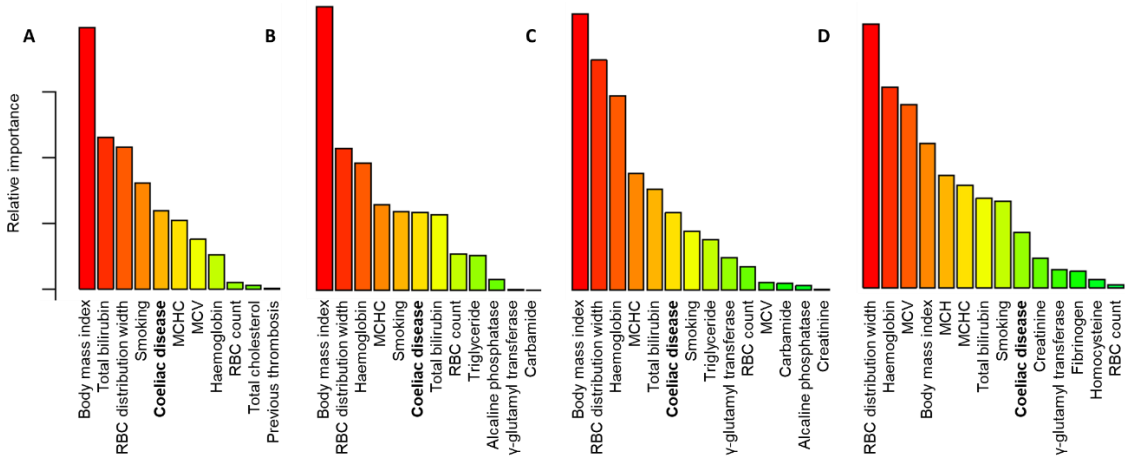


Fig 14. Important predictors of erythrocyte deformability represented by the elongation index at different shear stresses. Panels A, B, C and D show elongation indices at 30, 16.87, 9.49 and 5.33 Pa, respectively. The figures were generated with random forest analysis. We added 34 co-variates to the 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.

Table 9. 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 13. 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.

7.2.2 Natural anticoagulants

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 10). In line with these, random forest analysis did not highlight CeD as an important predictor of any of these outcomes.

Table 10. 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.

7.2.3 Homocysteine

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 (Fig 15).

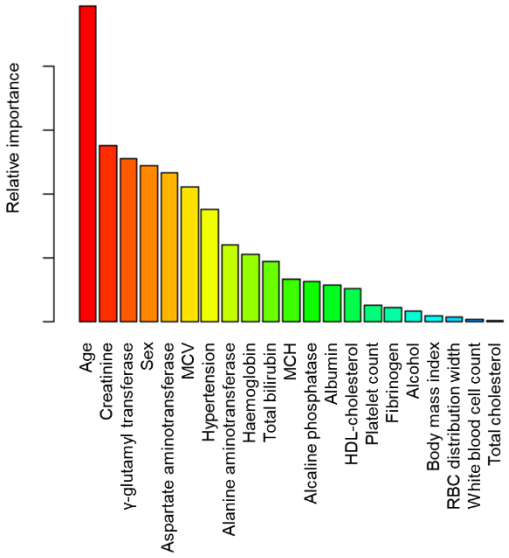


Fig 15. Important predictors of homocysteine level. The figure was generated with random forest analysis. We added 34 co-variates to the model, but only the important predictors of the outcome are shown. The relative importance is proportional to the height of the bars. The number of participants is 97 in the analysis. HDL: high-density lipoprotein; MCH: mean corpuscular haemoglobin; MCV: mean corpuscular volume; RBC: red blood cell. The figure is the author's own work.

7.3 The effects of dietary adherence

7.3.1 Haemorheological parameters

ED at high shear stresses was impaired both in CeD patients who were seropositive and seronegative (Fig 16A, solid green arrows) and both in CeD patients with poor and good dietary review through interview (Fig 16B, solid green arrows) compared to control subjects. However, ED at high shear stresses did not differ significantly between seropositive and seronegative CeD (Fig 16A, yellow dash line) or urine-GIP+ and urine-GIP− CeD patients (Fig 16C, 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 16B, 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 16D and 16E, yellow dash line). In contrast, urine-GIP+ CeD patients had significantly impaired ED compared to urine-GIP− CeD patients and control subjects (Fig 16F, solid green arrow), without a significant difference between urine-GIP− CeD patients and controls (Fig 16F, 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.

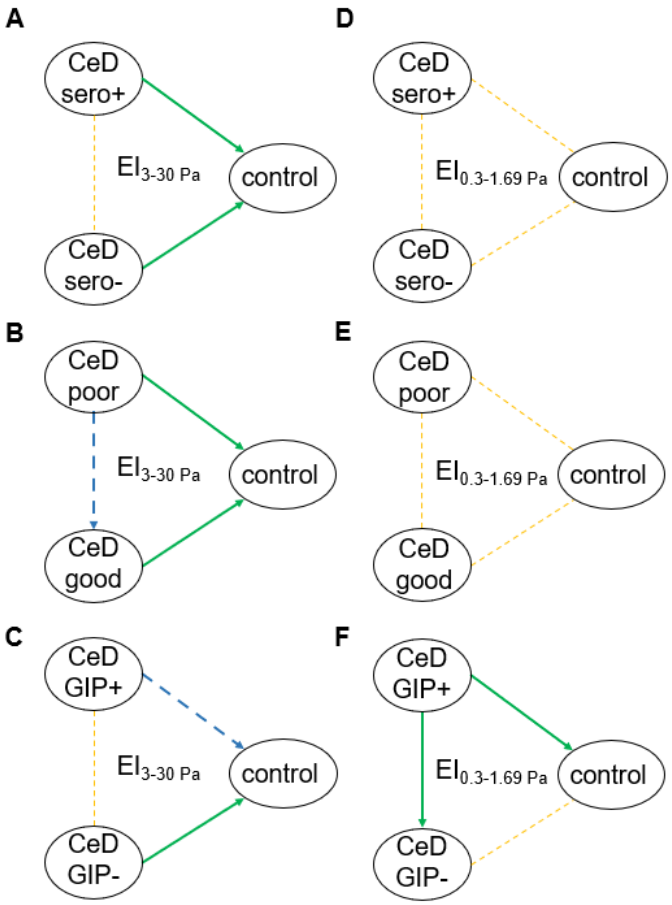


Fig 16. Model for the association of erythrocyte deformability with dietary adherence. Erythrocyte deformability is represented by the elongation index. **A** and **D**: coeliac-specific serology and erythrocyte deformability at high and low shear stresses, respectively. **B** and **E**: dietary review 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.

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 AI, $t_{1/2}$ and γ consistently (adjusted p-values <0.01 for all; for figure, see Fig 17 and Appendix 4), 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 (Appendix 4).

In random forest analysis, dietary adherence-related variables did not prove to be important predictors of the outcomes.

Table 11–13 show haemorheological parameters broken down to groups and dietary adherence estimated with CeD-specific serology, dietary review through interview and urine-GIP measurement.

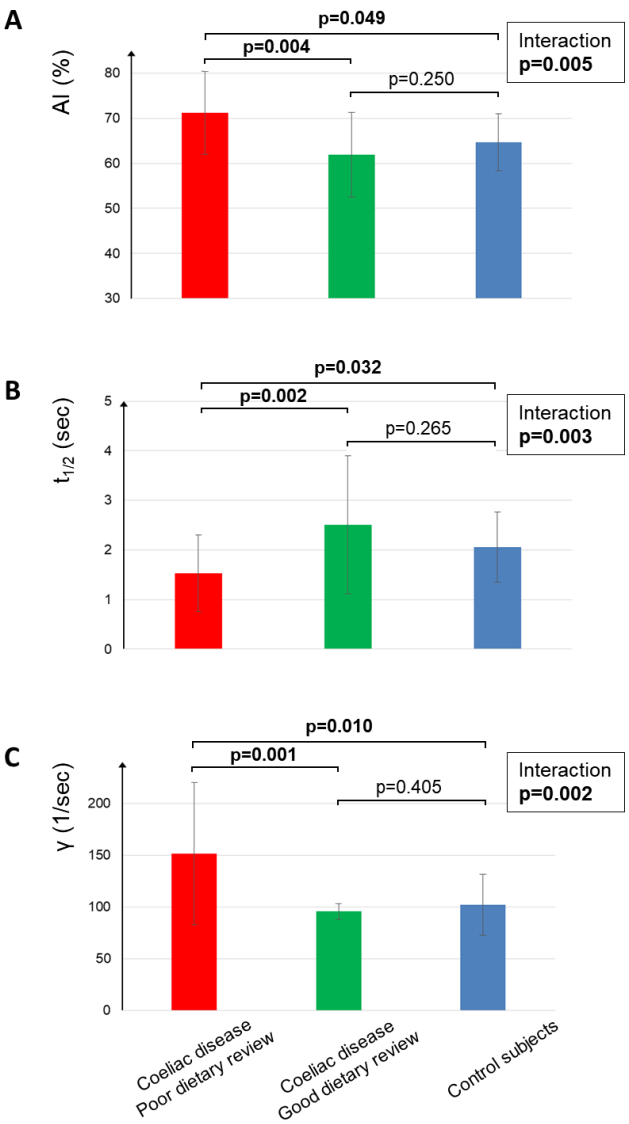


Fig 17. Erythrocyte aggregation and dietary review through interview. A: Aggregation index across groups. **B:** Aggregation half-time across groups. **C:** Threshold shear rate across group. P-values were generated with the one-way Analysis of Variance model after logarithmic transformation of the data in the case of $t_{1/2}$ and γ, 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; γ: threshold shear rate; AI: aggregation index. The figure is the author's own work.

7.3.2 Natural anticoagulants

Dividing patients by sero-positivity, 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.

7.3.3 Homocysteine

The level of homocysteine did not differ significantly across subgroups by different modalities of dietary adherence (p=0.107, p=0.114 and p=0.844 for the comparisons of subgroups by CeD-specific serology, dietary review through interview and urine-GIP detection, respectively).

Table 11. Dietary adherence estimated through coeliac-specific serology

	Seropositive CeD (n=14) (Group 1)	Seronegative CeD (n=36) (Group 2)	Control subjects (n=50) (Group 3)	P-values			
				Interaction	Group 1 vs 2	Group 1 vs 3	Group 2 vs 3
Haematocrit (%)	44.00±4.49	43.08±3.29	44.44±3.30	0.208			
Whole blood viscosity (mPa·sec)	4.17±0.57	3.99±0.36	4.14±0.43	0.266			
Plasma viscosity (mPa·sec)	1.24±0.16	1.24±0.16	1.27±0.15	0.456			
Fibrinogen (g/L)	2.83 [2.54–3.03]*	2.98 [2.66–3.78]	3.16 [2.71–3.59]*	0.263			
Erythrocyte aggregation							
AI (%)	63.63±11.53	63.79±9.54	64.60±6.33	0.879			
$T_{1/2}$ (sec)	2.36±1.28	2.29±1.39	2.06±0.71	0.917			
γ (1/sec)	115.36±75.27	103.54±36.82	102.25±29.62	0.996			
Erythrocyte deformability							
EI, 30.00 Pa	0.6250 [0.6163–0.6288]	0.6255 [0.6220–0.6293]	0.6290 [0.6260–0.6320]	**0.008**	1.000	**0.036**	**0.037**
EI, 16.87 Pa	0.5975 [0.5895–0.6035]	0.6000 [0.5965–0.6033]	0.6050 [0.6020–0.6060]	**0.001**	1.000	**0.006**	**0.006**
EI, 9.49 Pa	0.5555 [0.5463–0.5610]	0.5555 [0.5518–0.5600]	0.5610 [0.5563–0.5640]	**0.002**	1.000	**0.043**	**0.006**
EI, 5.33 Pa	0.5010 [0.4930–0.5075]	0.5015 [0.4958–0.5033]	0.5060 [0.5000–0.5100]	**0.010**	1.000	**0.010**	0.275
EI, 3.00 Pa	0.4290 [0.4223–0.4358]	0.4250 [0.4190–0.4310]	0.4310 [0.4260–0.4378]	**0.039**	0.999	**0.033**	1.000
EI, 1.69 Pa	0.3405 [0.3308–0.3513]	0.3340 [0.3283–0.3465]	0.3420 [0.3330–0.3470]	0.363			
EI, 0.95 Pa	0.2390 [0.2200–0.2448]	0.2260 [0.2158–0.2425]	0.2280 [0.2280–0.2448]	0.400			
EI, 0.53 Pa	0.1245 [0.1113–0.1398]	0.1165 [0.1038–0.1355]	0.1290 [0.1138–0.1365]	0.514			
EI, 0.30 Pa	0.0440 [0.0163–0.0550]	0.0370 [0.0070–0.0495]	0.0400 [0.0303–0.0505]	0.658			

Regarding p-values, boldface type indicates a statistically significant difference. *indicates unsuccessful measurement for one CeD patient and one control subject. Parameters are reported in median [Q_1–Q_3] or mean ± SD, depending on the distribution. The analysis was done either with one-way Analysis of Variance or with the Kruskal-Wallis test (with posthoc Mann-Whitney test). AI: aggregation index; CeD: coeliac disease; EI: elongation index.

Table 12. Dietary adherence estimated with dietary review through interview

	CeD with poor adherence (n=10) (Group 1)	CeD with good adherence (n=40) (Group 2)	Control subjects (n=50) (Group 3)	P-values			
				Interaction	Group 1 vs 2	Group 1 vs 3	Group 2 vs 3
Haematocrit (%)	42.10±3.81	43.65±3.58	44.44±3.30	0.133			
Whole blood viscosity (mPa·sec)	4.10±0.50	4.03±0.42	4.14±0.43	**0.003**	**0.004**	0.142	0.055
Plasma viscosity (mPa·sec)	1.38±0.28	1.21±0.09	1.27±0.15	0.466			
Fibrinogen (g/L)	3.62 [2.90–4.10]*	2.86 [2.67–3.49]	3.16 [2.71–3.59]*	0.213			
Erythrocyte aggregation							
AI (%)	71.20±9.22	61.89±9.41	64.60±6.33	**0.005**	**0.004**	**0.049**	0.250
$T_{1/2}$ (sec)	1.53±0.77	2.51±1.39	2.06±0.71	**0.003**	**0.002**	**0.032**	0.265
γ (1/sec)	151.75±68.82	95.63±37.45	102.25±29.62	**0.002**	**0.001**	**0.010**	0.405
Erythrocyte deformability							
EI, 30.00 Pa	0.6190 [0.6143–0.6240]	0.6265 [0.6235–0.6300]	0.6290 [0.6260–0.6320]	**<0.001**	**0.025**	**<0.001**	0.143
EI, 16.87 Pa	0.5915 [0.5875–0.5980]	0.6010 [0.5970–0.6040]	0.6050 [0.6020–0.6060]	**<0.001**	0.157	**0.001**	**0.012**
EI, 9.49 Pa	0.5505 [0.5438–0.5548]	0.5570 [0.5518–0.5610]	0.5610 [0.5563–0.5640]	**0.001**	0.270	**0.002**	**0.020**
EI, 5.33 Pa	0.4980 [0.4898–0.5020]	0.5020 [0.4960–0.5053]	0.5060 [0.5000–0.5100]	**0.005**	0.709	**0.020**	**0.043**
EI, 3.00 Pa	0.4275 [0.4148–0.4308]	0.4255 [0.4190–0.4350]	0.4310 [0.4260–0.4378]	0.054			
EI, 1.69 Pa	0.3405 [0.3233–0.3443]	0.3350 [0.3290–0.3493]	0.3420 [0.3330–0.3470]	0.428			
EI, 0.95 Pa	0.2410 [0.2113–0.2420]	0.2300 [0.2160–0.2440]	0.2280 [0.2280–0.2448]	0.583			
EI, 0.53 Pa	0.1325 [0.1030–0.1398]	0.1180 [0.1055–0.1373]	0.1290 [0.1138–0.1365]	0.574			
EI, 0.30 Pa	0.0495 [0.0135–0.0550]	0.0350 [0.0108–0.0510]	0.0400 [0.0303–0.0505]	0.500			

Regarding p-values, boldface type indicates a statistically significant difference. *indicates unsuccessful measurement for one CeD patient and one control subject. Parameters are reported in median [Q_1–Q_3] or mean ± SD, depending on the distribution. The analysis was done either with one-way Analysis of Variance (with posthoc Tukey test) or with the Kruskal-Wallis test (with posthoc Mann-Whitney test). AI: aggregation index; CeD: coeliac disease; EI: elongation index.

Table 13. Dietary adherence estimated through urine-GIP measurement

	GIP+ CeD (n=6) (Group 1)	GIP– CeD (n=44) (Group 2)	Control subjects (n=50) (Group 3)	P-values			
				Interaction	Group 1 vs 2	Group 1 vs 3	Group 2 vs 3
Haematocrit (%)	40.83±5.49	43.68±3.25	44.44±3.30	**0.049**	0.142	**0.044**	0.536
Whole blood viscosity (mPa·sec)	3.83±0.59	4.07±0.41	4.14±0.43	0.180			
Plasma viscosity (mPa·sec)	1.14±0.07	1.26±0.16	1.27±0.15	0.063			
Fibrinogen (g/L)	2.57 [2.46–2.59]*	2.95 [2.70–3.78]	3.16 [2.71–3.59]*	0.141			
Erythrocyte aggregation							
AI (%)	57.01±8.24	64.67±9.86	64.60±6.33	0.094			
$T_{1/2}$ (sec)	3.09±1.45	2.21±1.31	2.06±0.71	0.110			
γ (1/sec)	102.08±27.95	107.50±52.48	102.25±29.62	0.998			
Erythrocyte deformability							
EI, 30.00 Pa	0.6250 [0.6165–0.6268]	0.6255 [0.6218–0.6293]	0.6290 [0.6260–0.6320]	**0.008**	1.000	0.162	**0.016**
EI, 16.87 Pa	0.6010 [0.5858–0.6035]	0.5995 [0.5948–0.6033]	0.6050 [0.6020–0.6060]	**0.001**	1.000	0.301	**0.001**
EI, 9.49 Pa	0.5550 [0.5395–0.5593]	0,5555 [0.5500–0.5603]	0.5610 [0.5563–0.5640]	**0.002**	1.000	0.233	**0.003**
EI, 5.33 Pa	0.4965 [0.4838–0.5010]	0.5020 [0.4960–0.5050]	0.5060 [0.5000–0.5100]	**0.007**	0.986	0.071	**0.023**
EI, 3.00 Pa	0.4170 [0.4060–0.4235]	0.4260 [0.4198–0.4350]	0.4310 [0.4260–0.4378]	**0.010**	0.164	**0.016**	0.218
EI, 1.69 Pa	0.3210 [0.3123–0.3298]	0.3370 [0.3300–0.3503]	0.3420 [0.3330–0.3470]	**0.009**	**0.016**	**0.007**	1.000
EI, 0.95 Pa	0.2075 [0.1903–0.2143]	0.2380 [0.2178–0.2440]	0.2280 [0.2280–0.2448]	**0.005**	**0.006**	**0.004**	1.000
EI, 0.53 Pa	0.0910 [0.0660–0.0988]	0.1260 [0.1100–0.1405]	0.1290 [0.1138–0.1365]	**0.006**	**0.007**	**0.005**	1.000
EI, 0.30 Pa	–0.0260 [–0.0398 to –0.0025]	0.0405 [0.0255–0.0530]	0.0400 [0.0303–0.0505]	**0.003**	**0.002**	**0.003**	1.000

Regarding p-values, boldface type indicates a statistically significant difference. *indicates unsuccessful measurement for one CeD patient and one control subject. Parameters are reported in median [Q_1–Q_3] or mean ± SD, depending on the distribution. The analysis was done either with one-way Analysis of Variance (with posthoc Tukey test) or with the Kruskal-Wallis test (with posthoc Mann-Whitney test). AI: aggregation index; CeD: coeliac disease; EI: elongation index; GIP: gluten immunogenic peptide.

8. Discussion

8.1 Summary of findings

This study aimed to assess the haemorheological, natural anticoagulant and homocysteine 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. In contrast, we found a consistent shift in the parameters describing EA and WBV towards a prothrombotic direction in CeD patients with poor dietary review through interview compared to those with good dietary adherence. 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. Besides, these findings contribute to the understanding of the mechanism of increased CV risk in CeD despite the absence of traditional risk factors.

8.2 Explanation and elaboration

8.2.1 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. Low shear stresses (that is, 0.3-1.69 Pa) model the pressure conditions of the microcirculation, whereas high shear stresses rather model the pressure in the medium-large arteries (that is, 3-30 Pa). Although deformation does not necessarily affect all parts of the RBC equally as symmetric forces seldom occur in vivo, EI—calculated based on the short and long axes of the cells (Fig 13 inlet)—quantifies ED reliably. The higher the EI, the more significant the change in shape, so that the better the deformability. In our study, we found lower EI in CeD compared to control subjects, but the level of statistical significance was attained only at high shear stresses. The lower the shear stress, the higher the measure of dispersion (as shown by the error bars in Fig 13), which resulted in remarkably low statistical power at low shear stresses (<10% for $EI_{0.3Pa}$), raising concerns about the β-

type error. The high dispersion is responsible for the surprisingly high number of patients which would have been required for the second phase of the study (for details, see subchapter 6.9).

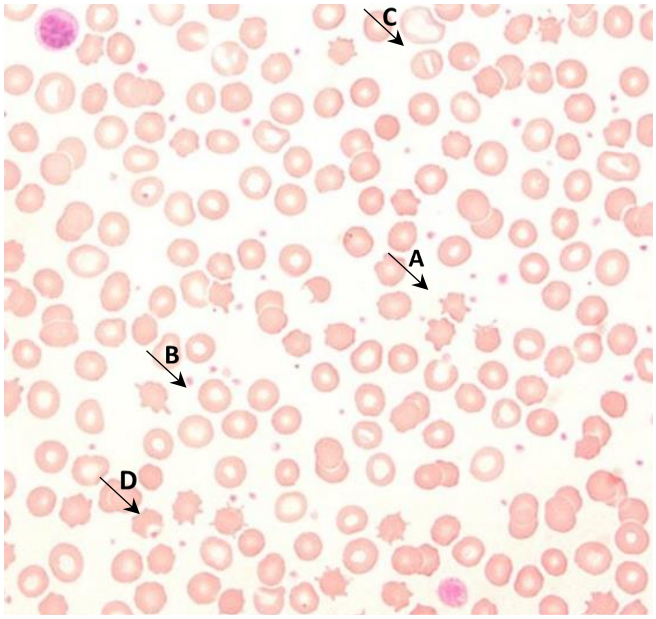


Fig 18. Blood film of a patient with functional hyposplenism. The film shows acanthocytes (arrow A), target cells (arrow B), stomatocytes (arrow C) and Howell-Jolly bodies (arrow D). Source: https://imagebank.hematology.org/

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[135], the spleen no longer removes old RBCs from the circulation (Fig 18)[136]. As a result, defective, rigid cells accumulate. The splenic function might improve during a GFD (if started at early stages)[137], which, at least partly, explains our results. (2) Increased oxidative stress and reduced nitrogen oxide production (e.g. by gluten or its derivatives) can serve as another explanation. A strict GFD mitigates oxidative stress in CeD[101]. (3) In line with the earlier data[138], RBC distribution width was significantly higher in CeD compared to the control group in our study. Anisocytosis can be the consequence of the complex deficiency of iron, vitamin B_{12} and other micronutrients in CeD and is expected to improve during a GFD[138]. The greater the variation in RBC distribution width, the lower the EI[139]. (4) Comorbid conditions (e.g. components of metabolic syndrome including hypertension, obesity, diabetes mellitus and dyslipidaemia) can affect ED as well (for a review on erythrocyte abnormalities in metabolic syndrome, see the paper of Gyawali et al.[140]). Although most between-group differences were diminished by matching, immune-mediated comorbidities occurred more frequently among CeD patients compared to control subjects, which difference may explain impaired ED with CeD (for details about haemorheological alterations in immune-mediated diseases, see subchapter 4.8.6; for the list of comorbidities of the study participants, see Appendix 1).

Membrane cholesterol content determines membrane fluidity, thereby affecting deformability: the more cholesterol in the membrane, the more rigid the RBCs are (i.e. the relationship between ED and membrane cholesterol content is inverse)[141]. In our

study, CeD patients had lower EI (i.e. impaired ED) despite having a lower cholesterol level, opposing the expectations and strengthening the causative role of CeD in modifying ED. It also implies that, from a haemorheological point of view, maintaining a lower cholesterol level should be considered optimal in CeD, because elevated cholesterol is expected to deteriorate ED further.

As we did not observe any difference between the seropositive and seronegative groups and both had lower EI compared to control subjects, we can conclude that the impairment of ED at high shear stresses is independent on the EMA/TGA-mediated immune response. CeD patients with good dietary review had higher EI (i.e. better ED) only at 30 Pa shear stress compared to those with poor dietary review, which means that long-term gluten intake—independently on EMA/TGA-mediated immune response—has only limited effect on ED. In contrast, urine-GIP+ CeD patients had worse ED compared to both urine-GIP– CeD patients and controls. These associations imply that gluten intake can, e.g. via oxidative stress[101,142] or non-EMA/TGA-dependent immunological mechanisms, modify ED. In summary, the impairment of ED is only partly dependent on a GFD in CeD.

Studies on the CV involvement of ED are available in a high number but, usually, the size of the study population, control for covariates and the length of follow-up are limited. Impaired ED can be measured shortly after acute myocardial infarction or unstable angina[143-145], and within 1[143,144] and 3 years after acute myocardial infarction[146]; whereas changes in ED during follow-up after an acute CV event seem unremarkable[144,145]. ED predicts the success of mechanical reperfusion[147] or even the development of severe CV events 3 years after acute myocardial infarction[148]. Impaired ED is detectable shortly[149] and 2 years after stroke as well[150]. The overall evidence suggests that the impairment of ED precedes the acute events (and is not their consequence). 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 a nutrient profile of a GFD (hypercalorigenic, rich in carbohydrate and fat, poor in fibre; detailed in subchapter 4.4.3) being unfavourable to the development of CV risk factors, particularly to obesity and dyslipidaemia[61,62]. ED is extensively studied in obesity, particularly in morbidly obese patients subjected to bariatric surgery[151-153]. Whether the effect of obesity is independent of or mediated by other

factors has remained undecided: a study found waist circumference to be an independent predictor of EI[153], whereas another study concluded that other component(s) of metabolic syndrome (likely the insulin resistance) is guilty of impairing ED[154]. In CeD, the levels of total, LDL- and HDL-cholesterol tend to increase, not only due to improved absorption but also due to the high intake of fatty acids, during a GFD[61]. The importance of cholesterol in determining ED has been discussed above. In addition to regular exercise, preventive strategies should focus on education to improve nutritional awareness to optimize body weight and blood lipids. The consumption of naturally gluten-free foods—fruits, vegetables, dairy products, lean meat—should be encouraged instead of the hypercalorigenic gluten-free grain products, chocolate and other sweet foods.

Randomized controlled trials investigated if pharmacological interventions can improve ED. Notably, most trialists attributed the beneficial effect of the intervention to lipid-lowering. The efficacy of statins has been proven in various study populations, e.g. type II diabetes mellitus[155], hyperlipidaemia[156] and cerebrovascular diseases[157]. The pharmacological control of hypertension[158] and diabetes mellitus[159], comorbidities impairing ED, can be beneficial as well. Besides, the effects of many other nutritional supplements, e.g. resveratrol[160] and ω-3 fatty acids[161], are promising. Even eating dark chocolate improves ED[162].

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. Measured with LORCA, we can describe EA with three parameters: AI, $t_{1/2}$ and γ, each covering a different aspect of aggregation. 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 (higher AI and γ, lower $t_{1/2}$) 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.

Since EA is mostly dependent on plasma proteins, we thought that fibrinogen increased reactively to gluten intake and is, thereby, responsible for the increased aggregation. Measuring the level of fibrinogen, we did not identify a significant difference between the groups. As there was no difference between seropositive and seronegative CeD patients in EA, the EMA/TGA-mediated immune response is unlikely in the background of the increased EA. We suspect the causative role of other antibodies or inflammatory proteins[163], not measured in this study.

Studies reporting on EA and CV involvement are limited by similar factors discussed above concerning ED. EA increases shortly after acute coronary syndrome[145,164,165], including acute myocardial infarction[166,167] as well as in the long-term[165]. In contrast to ED, alteration of EA after acute myocardial infarction seems to be time-dependent[168]. EA increases in the acute phase of stroke as well[149,166]. The increased EA can be manifested in elevated WBV in patients with poor dietary adherence, as measured in our study as well (the similar levels of cell counts, haematocrit and other plasma proteins—the main determinants of WBV—between the groups support this theory). The alteration of WBV, the most important haemorheological parameter, has far-reaching consequences. WBV (and PV) are extensively studied in a large variety of CV diseases, including coronary heart disease[169]. Large cohort studies, such as the Edinburgh Artery Study[170], the MONICA-Augsburg Cohort Study[171] and the West of Scotland Coronary Prevention Study[172] provided (sometimes, conflicting) results on the predictive role of WBV (and haematocrit, fibrinogen, PV) in the development of major CV events, myocardial infarction and stroke in co-variate adjusted analyses. Besides, these parameters correlate with clinical outcomes, e.g. success of mechanical reperfusion[147], development of stent thrombosis[173] and that of recurrent in-hospital and long-term major CV events[174]. These data indicate that 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.

As mentioned in subchapter 4.8.5, several studies identified haemorheological alterations in inflammatory bowel disease, worth being contrasted to our findings as both bowel diseases are associated with heavy chronic inflammation and malabsorption. Although the tendencies in inflammatory bowel disease are similar to that we observed in CeD about EA and ED, the activity-dependent elevation of PV—probably resulting from hyperfibrinogenaemia, not observed in CeD—imposes an additional risk of thrombotic events.

8.2.2 Natural anticoagulants

Natural anticoagulants are more important determinants of venous rather than arterial thrombotic events, in which abnormalities of the arterial wall are decisive. Hereditary protein C, protein S and antithrombin deficiency increase the risk of VTE to 7-fold, 5–11.5-fold and 2.2–8.1-fold, respectively[175]. Case reports suggested a link

between the decreased activity of natural anticoagulants and the development of myocardial infarction and stroke, which theory has remained unconfirmed in controlled studies yet[175].

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, opposing previous evidence from case reports and case series[97-99]. These findings are in line with the observation that a 1-year GFD recovers regular vitamin K status[113]; therefore, the synthesis of protein C and protein S normalizes. Note that the dispersion was high in the sample, which, again, raises concerns about the β-type error. However, even if the level of significance had been attained ($p<0.05$), the difference between the groups would have been clinically negligible (–9.7% for the activity of protein C and –5.6% for that of protein S, favouring the control group). These findings corroborated with our observations on the activity of antithrombin.

The use of oral contraceptives was balanced between the groups (8 vs 10 users in CeD vs control groups, respectively); however, we could not take this into account in the multivariate analysis as the study population was not great enough to construct a separate stratum for females.

8.2.3 Homocysteine

An increase in the level of homocysteine is a robust and independent predictor of ischaemic stroke[176] and myocardial infarction[177]. CeD patients at diagnosis exhibit a higher level of homocysteine, compared to control subjects[91,104]. However, studies resulted in divergent results as to whether the level of homocysteine reduces[91,178] or continues to remain elevated[90,104,114] 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.

Vitamin B_6 and folate status, but not CeD, are independent predictors of the level of homocysteine[94]. Although we do not have direct evidence on that a strict GFD with vitamin supplementation results in long-term clinical benefits, administration of vitamin B_6 and folate (maybe vitamin B_{12} as well) might improve hyperhomocysteinaemia.

8.3 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. Published pre-study protocols help to avoid reporting bias.
(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 (e.g. sampling after the consumption of half-litre water, sample processing within 2 hours).
(7) A complex analytical approach was developed and executed by a skilled statistician.

Nevertheless, we must acknowledge that the study has **several limitations**.

(1) The main limitation is the study design: 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, as recorded in the pre-study protocol, 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. Of note, patients on a long-term GFD prevailed.
(4) The limited sample size risks β-type error, particularly in the comparison of sub-groups by dietary adherence.
(5) As per the current guidelines, we do not take follow-up biopsies routinely, so that the association between the mucosal status, reflecting the activity of the inflammation, and the outcomes was not studied.
(6) The few pieces of missing data regarding the explanatory variables and the secondary outcomes limited the use of multivariate analysis.
(7) We did not achieve a perfect match for each case due to the limited sample size of the control group; therefore, we matched within ranges of tolerance (for details, see subchapter 6.9). Ideally, propensity-matching by several co-variates would have made the study groups almost entirely similar by characteristics. However, doing so

requires approximately 2–3 times more control subjects than we had (surprisingly, we encountered enormous difficulties when aiming to recruit eligible control subject—free from acute and advanced chronic diseases—from the gastroenterology outpatient clinic).

(8) The random forest model quantifies the relative but not the absolute effect of the predictors. We tried to build multivariate regression models for quantification of the independent effect of the variables; however, all proved to be unstable, probably due to the many explanatory variables.

8.4 Generalizability of the findings

The study population included relatively young—on average, 40 years of age at inclusion—CeD patients, with a strong female predominance. Likely due to the strict eligibility criteria, both CeD patients and control subjects had few and relatively mild comorbidities, including only two cases of prior arterial and venous thrombotic events. The majority of the study population was at low to moderate CV risk. Besides, 47 of 50 CeD patients were on a GFD ≥1 year (with a median 5.5 years), 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.

9. Summary of novel findings and perspectives

9.1 Summary of novel findings

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 19). 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.

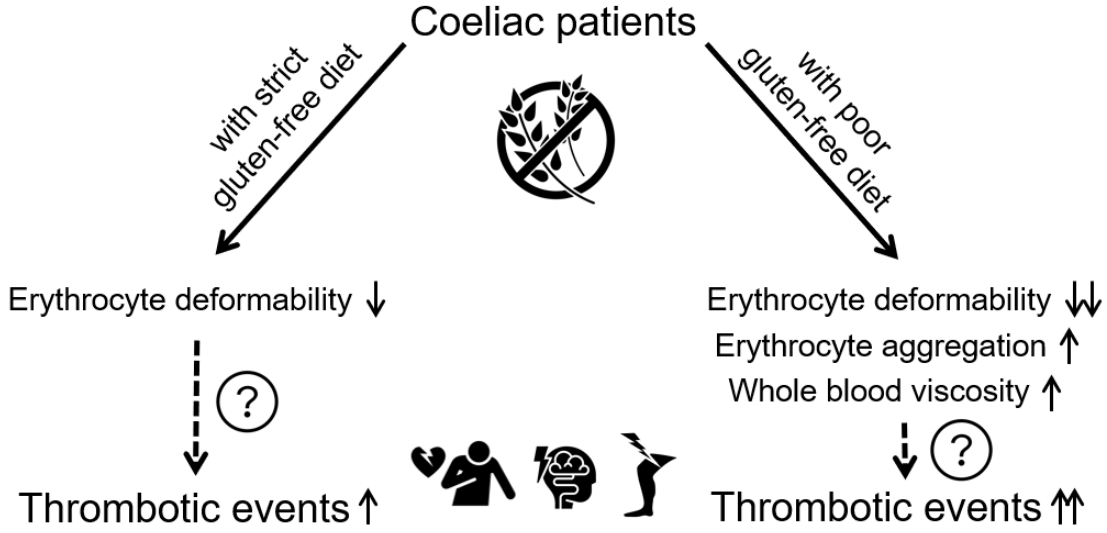


Fig 19. Summary of prothrombotic haemorheological changes among coeliac patients compared to control

9.2 Future perspectives

Further studies are needed to

1. verify our findings in a prospective cohort study recruiting cases immediately after diagnosis of CeD and repeating the measurements later during a GFD;
2. explore the mechanisms leading to impaired ED and by which a GFD improves haemorheological profile in CeD;
3. investigate the association between haemorheological parameters and CV events specifically in CeD;
4. test if pharmacological and non-pharmacological interventions, such as anti-oxidants, statins or lifestyle changes, can restore or improve ED in CeD.

10. Acknowledgements

First and foremost, thanks to **dr Judit Bajor** for constant guidance and support provided in this and many other projects, and outside the work during the past three years.

Thanks to **dr Péter Hegyi** for providing the opportunity of initiating this study and for constructive criticism when interpreting the results. Besides, I am grateful to him for driving my scientific carrier and for his never-ending optimism, which encouraged everyone in his surroundings, myself included, to set higher standards.

Thanks to all peers who contributed to any phase of the study. Although many worked on this project, first, let me express my gratitude to the **multidisciplinary staff of the Institute for Translational Medicine**, who assisted me in planning, writing and publishing the study protocol, in the recruitment of study participants, in data management, in statistical analysis and in the publication of the papers. Thanks to the site of recruitment, **Division of Gastroenterology, First Department of Medicine**, Medical School, University of Pécs; and to student researcher **Mátyás Nagy**, who merits to be highlighted for assisting in the project.

Thanks to the employees of the **Haemorheological Laboratory, First Department of Medicine**, Medical School, University of Pécs, who performed the haemorheological measurements. Besides, thanks to the employees of the **Department of Laboratory Medicine** and those of the **Department of Immunology and Biotechnology**, Medical School, University of Pécs, who performed the immunological and other the study-related measurements.

Research projects cannot be initiated and executed without financial and human resources. Study and centre costs were covered by the **Medical School, University of Pécs** and by the **Translational Medicine Foundation**. In addition, the project was supported by the New National Excellence Programme, Ministry of Human Capacities (ÚNKP-17-3-II, ÚNKP-18-3-I and ÚNKP-19-3-I), by an Economic Development and Innovation Operative Programme Grant (GINOP 2.3.2.-15-2016-00048) and a Human Resources Development Operative Programme Grant (EFOP-3.6.2-16-2017-00006) of the National Research, Development and Innovation Office, Hungary. I hereby declare that funders have no influence on preparations, course, interpretation or publication of results.

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

11. References

1. Singh P, Arora A, Strand TA, et al. Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol.* 2018;16(6):823-836.e822. DOI: 10.1016/j.cgh.2017.06.037.
2. Myléus A, Ivarsson A, Webb C, et al. Celiac disease revealed in 3% of Swedish 12-year-olds born during an epidemic. *J Pediatr Gastroenterol Nutr.* 2009;49(2):170-176. DOI: 10.1097/MPG.0b013e31818c52cc.
3. Fukunaga M, Ishimura N, Abe T, et al. Serological screening for celiac disease in adults in Japan: Shimane CoHRE study. *JGH Open.* 2020;4(4):558-560. DOI: 10.1002/jgh3.12334.
4. Korponay-Szabó IR, Kovács JB, Czinner A, Gorácz G, Vámos A, Szabó T. High prevalence of silent celiac disease in preschool children screened with IgA/IgG antiendomysium antibodies. *J Pediatr Gastroenterol Nutr.* 1999;28(1):26-30. DOI: 10.1097/00005176-199901000-00008.
5. Korponay-Szabó IR, Szabados K, Pusztai J, et al. Population screening for coeliac disease in primary care by district nurses using a rapid antibody test: diagnostic accuracy and feasibility study. *BMJ.* 2007;335(7632):1244. DOI: 10.1136/bmj.39405.472975.80.
6. King JA, Jeong J, Underwood FE, et al. Incidence of Celiac Disease Is Increasing Over Time: A Systematic Review and Meta-analysis. *Am J Gastroenterol.* 2020;115(4):507-525. DOI: 10.14309/ajg.0000000000000523.
7. Bach JF. The hygiene hypothesis in autoimmunity: the role of pathogens and commensals. *Nat Rev Immunol.* 2018;18(2):105-120. DOI: 10.1038/nri.2017.111.
8. Biesiekierski JR. What is gluten? *J Gastroenterol Hepatol.* 2017;32 Suppl 1:78-81. DOI: 10.1111/jgh.13703.
9. Cebolla Á, Moreno MdL, Coto L, Sousa C. Gluten Immunogenic Peptides as Standard for the Evaluation of Potential Harmful Prolamin Content in Food and Human Specimen. *Nutrients.* 2018;10(12):1927. DOI: 10.3390/nu10121927.
10. Ciccocioppo R, Di Sabatino A, Corazza GR. The immune recognition of gluten in coeliac disease. *Clin Exp Immunol.* 2005;140(3):408-416. DOI: 10.1111/j.1365-2249.2005.02783.x.
11. Greco L, Romino R, Coto I, et al. The first large population based twin study of coeliac disease. *Gut.* 2002;50(5):624-628. DOI: 10.1136/gut.50.5.624.

12. Rubio-Tapia A, Van Dyke CT, Lahr BD, et al. Predictors of family risk for celiac disease: a population-based study. *Clin Gastroenterol Hepatol.* 2008;6(9):983-987. DOI: 10.1016/j.cgh.2008.04.008.
13. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. *J Exp Med.* 1989;169(1):345-350. DOI: 10.1084/jem.169.1.345.
14. Al-Hussaini A, Alharthi H, Osman A, Eltayeb-Elsheikh N, Chentoufi A. Genetic susceptibility for celiac disease is highly prevalent in the Saudi population. *Saudi J Gastroenterol.* 2018;24(5):268-273. DOI: 10.4103/sjg.SJG_551_17.
15. Megiorni F, Pizzuti A. HLA-DQA1 and HLA-DQB1 in Celiac disease predisposition: practical implications of the HLA molecular typing. *J Biomed Sci.* 2012;19(1):88-88. DOI: 10.1186/1423-0127-19-88.
16. Vader W, Stepniak D, Kooy Y, et al. The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses. *Proc Natl Acad Sci U S A.* 2003;100(21):12390-12395. DOI: 10.1073/pnas.2135229100.
17. 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. DOI: 10.1371/journal.pone.0212329.
18. 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. DOI: 10.1111/iji.12415.
19. Al-Toma A, Volta U, Auricchio R, et al. European Society for the Study of Coeliac Disease (ESsCD) guideline for coeliac disease and other gluten-related disorders. *United European Gastroenterol J.* 2019;7(5):583-613. DOI: 10.1177/2050640619844125.
20. Wolters VM, Wijmenga C. Genetic background of celiac disease and its clinical implications. *Am J Gastroenterol.* 2008;103(1):190-195. DOI: 10.1111/j.1572-0241.2007.01471.x.
21. Garner CP, Murray JA, Ding YC, Tien Z, van Heel DA, Neuhausen SL. Replication of celiac disease UK genome-wide association study results in a US population. *Human molecular genetics.* 2009;18(21):4219-4225. DOI: 10.1093/hmg/ddp364.

22. Fasano A, Catassi C. Clinical practice. Celiac disease. *N Engl J Med.* 2012;367(25):2419-2426. DOI: 10.1056/NEJMcp1113994.
23. Fasano A, Not T, Wang W, et al. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet (London, England).* 2000;355(9214):1518-1519. DOI: 10.1016/s0140-6736(00)02169-3.
24. Ludvigsson JF, Leffler DA, Bai JC, et al. The Oslo definitions for coeliac disease and related terms. *Gut.* 2013;62(1):43-52. DOI: 10.1136/gutjnl-2011-301346.
25. Dominguez Castro P, Harkin G, Hussey M, et al. Changes in Presentation of Celiac Disease in Ireland From the 1960s to 2015. *Clin Gastroenterol Hepatol.* 2017;15(6):864-871.e863. DOI: 10.1016/j.cgh.2016.11.018.
26. Pallav K, Xu H, Leffler DA, Kabbani T, Kelly CP. Immunoglobulin A deficiency in celiac disease in the United States. *J Gastroenterol Hepatol.* 2016;31(1):133-137. DOI: 10.1111/jgh.13176.
27. Balaban DV, Popp A, Vasilescu F, Haidautu D, Purcarea RM, Jinga M. Diagnostic yield of endoscopic markers for celiac disease. *J Med Life.* 2015;8(4):452-457. DOI: not available.
28. Kurien M, Evans KE, Hopper AD, Hale MF, Cross SS, Sanders DS. Duodenal bulb biopsies for diagnosing adult celiac disease: is there an optimal biopsy site? *Gastrointest Endosc.* 2012;75(6):1190-1196. DOI: 10.1016/j.gie.2012.02.025.
29. Husby S, Koletzko S, Korponay-Szabó I, et al. European Society Paediatric Gastroenterology, Hepatology and Nutrition Guidelines for Diagnosing Coeliac Disease 2020. *J Pediatr Gastroenterol Nutr.* 2020;70(1):141-156. DOI: 10.1097/mpg.0000000000002497.
30. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology.* 1992;102(1):330-354. DOI: 10.1016/0016-5085(92)91819-P.
31. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol.* 1999;11(10):1185-1194. DOI: 10.1097/00042737-199910000-00019.
32. Meeuwisse GW. Diagnostic criteria in coeliac disease. *Acta Paediatr Scand.* 1970;59:461-463. DOI: not available.
33. Husby S, Koletzko S, Korponay-Szabó IR, et al. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of

coeliac disease. *J Pediatr Gastroenterol Nutr.* 2012;54(1):136-160. DOI: 10.1097/MPG.0b013e31821a23d0.

34. Rubio-Tapia A, Hill ID, Kelly CP, Calderwood AH, Murray JA, American College of G. ACG clinical guidelines: diagnosis and management of celiac disease. *Am J Gastroenterol.* 2013;108(5):656-677. DOI: 10.1038/ajg.2013.79.
35. Sarna VK, Lundin KEA, Mørkrid L, Qiao SW, Sollid LM, Christophersen A. HLA-DQ-Gluten Tetramer Blood Test Accurately Identifies Patients With and Without Celiac Disease in Absence of Gluten Consumption. *Gastroenterology.* 2018;154(4):886-896.e886. DOI: 10.1053/j.gastro.2017.11.006.
36. Singh RS, Singh A, Batra G, Kaur H, Medhi B. Novel targets for drug discovery in celiac disease. *Indian J Pharmacol.* 2019;51(5):359-365. DOI: 10.4103/ijp.IJP_679_19.
37. Murray JA, Watson T, Clearman B, Mitros F. Effect of a gluten-free diet on gastrointestinal symptoms in celiac disease. *Am J Clin Nutr.* 2004;79(4):669-673. DOI: 10.1093/ajcn/79.4.669.
38. Pinto-Sánchez MI, Causada-Calo N, Bercik P, et al. Safety of Adding Oats to a Gluten-Free Diet for Patients With Celiac Disease: Systematic Review and Meta-analysis of Clinical and Observational Studies. *Gastroenterology.* 2017;153(2):395-409.e393. DOI: 10.1053/j.gastro.2017.04.009.
39. Hischenhuber C, Crevel R, Jarry B, et al. Review article: safe amounts of gluten for patients with wheat allergy or coeliac disease. *Aliment Pharmacol Ther.* 2006;23(5):559-575. DOI: 10.1111/j.1365-2036.2006.02768.x.
40. *Codex standard for foods for special dietary use for persons intolerant to gluten.* Codex Alimentarius Commission; 2008.
41. Abu-Janb N, Jaana M. Facilitators and barriers to adherence to gluten-free diet among adults with celiac disease: a systematic review. *J Hum Nutr Diet.* 2020. DOI: 10.1111/jhn.12754.
42. Chauhan JC, Kumar P, Dutta AK, Basu S, Kumar A. Assessment of dietary compliance to gluten free diet and psychosocial problems in Indian children with celiac disease. *Indian J Pediatr.* 2010;77(6):649-654. DOI: 10.1007/s12098-010-0092-3.
43. Moreno ML, Rodríguez-Herrera A, Sousa C, Comino I. Biomarkers to Monitor Gluten-Free Diet Compliance in Celiac Patients. *Nutrients.* 2017;9(1). DOI: 10.3390/nu9010046.

44. Szakács Z, Mátrai P, Hegyi P, et al. Younger age at diagnosis predisposes to mucosal recovery in celiac disease on a gluten-free diet: A meta-analysis. *PLoS One.* 2017;12(11):e0187526-e0187526. DOI: 10.1371/journal.pone.0187526.

45. Kaukinen K, Peräaho M, Lindfors K, et al. Persistent small bowel mucosal villous atrophy without symptoms in coeliac disease. *Aliment Pharmacol Ther.* 2007;25(10):1237-1245. DOI: 10.1111/j.1365-2036.2007.03311.x.

46. Nachman F, Sugai E, Vázquez H, et al. Serological tests for celiac disease as indicators of long-term compliance with the gluten-free diet. *Eur J Gastroenterol Hepatol.* 2011;23(6):473-480. DOI: 10.1097/MEG.0b013e328346e0f1.

47. Silvester JA, Kurada S, Szwajcer A, Kelly CP, Leffler DA, Duerksen DR. Tests for Serum Transglutaminase and Endomysial Antibodies Do Not Detect Most Patients With Celiac Disease and Persistent Villous Atrophy on Gluten-free Diets: a Meta-analysis. *Gastroenterology.* 2017;153(3):689-701.e681. DOI: 10.1053/j.gastro.2017.05.015.

48. Li M, Yu L, Tiberti C, et al. A report on the International Transglutaminase Autoantibody Workshop for Celiac Disease. *Am J Gastroenterol.* 2009;104(1):154-163. DOI: 10.1038/ajg.2008.8.

49. Biagi F, Bianchi PI, Marchese A, et al. A score that verifies adherence to a gluten-free diet: a cross-sectional, multicentre validation in real clinical life. *Br J Nutr.* 2012;108(10):1884-1888. DOI: 10.1017/s0007114511007367.

50. Johansson K, Norström F, Nordyke K, Myleus A. Celiac Dietary Adherence Test simplifies Determining Adherence to a Gluten-free Diet in Swedish Adolescents. *J Pediatr Gastroenterol Nutr.* 2019;69(5):575-580. DOI: 10.1097/mpg.0000000000002451.

51. Silvester JA, Graff LA, Rigaux L, Walker JR, Duerksen DR. Symptomatic suspected gluten exposure is common among patients with coeliac disease on a gluten-free diet. *Aliment Pharmacol Ther.* 2016;44(6):612-619. DOI: 10.1111/apt.13725.

52. Comino I, Segura V, Ortigosa L, et al. Prospective longitudinal study: use of faecal gluten immunogenic peptides to monitor children diagnosed with coeliac disease during transition to a gluten-free diet. *Aliment Pharmacol Ther.* 2019;49(12):1484-1492. DOI: 10.1111/apt.15277.

53. Moreno ML, Cebolla Á, Muñoz-Suano A, et al. Detection of gluten immunogenic peptides in the urine of patients with coeliac disease reveals transgressions in the

gluten-free diet and incomplete mucosal healing. *Gut.* 2017;66(2):250-257. DOI: 10.1136/gutjnl-2015-310148.

54. Vici G, Belli L, Biondi M, Polzonetti V. Gluten free diet and nutrient deficiencies: A review. *Clin Nutr.* 2016;35(6):1236-1241. DOI: 10.1016/j.clnu.2016.05.002.
55. Sue A, Dehlsen K, Ooi CY. Paediatric Patients with Coeliac Disease on a Gluten-Free Diet: Nutritional Adequacy and Macro- and Micronutrient Imbalances. *Curr Gastroenterol Rep.* 2018;20(1):2. DOI: 10.1007/s11894-018-0606-0.
56. Fry L, Madden AM, Fallaize R. An investigation into the nutritional composition and cost of gluten-free versus regular food products in the UK. *J Hum Nutr Diet.* 2018;31(1):108-120. DOI: 10.1111/jhn.12502.
57. Kulai T, Rashid M. Assessment of Nutritional Adequacy of Packaged Gluten-free Food Products. *Can J Diet Pract Res.* 2014;75(4):186-190. DOI: 10.3148/cjdpr-2014-022.
58. Miranda J, Lasa A, Bustamante MA, Churruca I, Simon E. Nutritional differences between a gluten-free diet and a diet containing equivalent products with gluten. *Plant Foods Hum Nutr.* 2014;69(2):182-187. DOI: 10.1007/s11130-014-0410-4.
59. Zuccotti G, Fabiano V, Dilillo D, Picca M, Cravidi C, Brambilla P. Intakes of nutrients in Italian children with celiac disease and the role of commercially available gluten-free products. *J Hum Nutr Diet.* 2013;26(5):436-444. DOI: 10.1111/jhn.12026.
60. Babio N, Alcázar M, Castillejo G, et al. Patients With Celiac Disease Reported Higher Consumption of Added Sugar and Total Fat Than Healthy Individuals. *J Pediatr Gastroenterol Nutr.* 2017;64(1):63-69. DOI: 10.1097/mpg.0000000000001251.
61. Potter MDE, Brienesse SC, Walker MM, Boyle A, Talley NJ. Effect of the gluten-free diet on cardiovascular risk factors in patients with coeliac disease: A systematic review. *J Gastroenterol Hepatol.* 2018;33(4):781-791. DOI: 10.1111/jgh.14039.
62. Valvano M, Longo S, Stefanelli G, Frieri G, Viscido A, Latella G. Celiac Disease, Gluten-Free Diet, and Metabolic and Liver Disorders. *Nutrients.* 2020;12(4):940. DOI: 10.3390/nu12040940.
63. Verbeek WH, Van De Water JM, Al-Toma A, Oudejans JJ, Mulder CJ, Coupé VM. Incidence of enteropathy--associated T-cell lymphoma: a nation-wide study

of a population-based registry in The Netherlands. *Scand J Gastroenterol.* 2008;43(11):1322-1328. DOI: 10.1080/00365520802240222.

64. Askling J, Linet M, Gridley G, Halstensen TS, Ekström K, Ekbom A. Cancer incidence in a population-based cohort of individuals hospitalized with celiac disease or dermatitis herpetiformis. *Gastroenterology.* 2002;123(5):1428-1435. DOI: 10.1053/gast.2002.36585.

65. Viljamaa M, Kaukinen K, Pukkala E, Hervonen K, Reunala T, Collin P. Malignancies and mortality in patients with coeliac disease and dermatitis herpetiformis: 30-year population-based study. *Dig Liver Dis.* 2006;38(6):374-380. DOI: 10.1016/j.dld.2006.03.002.

66. Malamut G, Afchain P, Verkarre V, et al. Presentation and long-term follow-up of refractory celiac disease: comparison of type I with type II. *Gastroenterology.* 2009;136(1):81-90. DOI: 10.1053/j.gastro.2008.09.069.

67. Al-Toma A, Verbeek WH, Hadithi M, von Blomberg BM, Mulder CJ. Survival in refractory coeliac disease and enteropathy-associated T-cell lymphoma: retrospective evaluation of single-centre experience. *Gut.* 2007;56(10):1373-1378. DOI: 10.1136/gut.2006.114512.

68. Rubio-Tapia A, Kelly DG, Lahr BD, Dogan A, Wu TT, Murray JA. Clinical staging and survival in refractory celiac disease: a single center experience. *Gastroenterology.* 2009;136(1):99-107; quiz 352-103. DOI: 10.1053/j.gastro.2008.10.013.

69. Cottone M, Termini A, Oliva L, et al. Mortality and causes of death in celiac disease in a Mediterranean area. *Dig Dis Sci.* 1999;44(12):2538-2541. DOI: 10.1023/a:1026655609906.

70. Corrao G, Corazza GR, Bagnardi V, et al. Mortality in patients with coeliac disease and their relatives: a cohort study. *Lancet (London, England).* 2001;358(9279):356-361. DOI: 10.1016/s0140-6736(01)05554-4.

71. Peters U, Askling J, Gridley G, Ekbom A, Linet M. Causes of death in patients with celiac disease in a population-based Swedish cohort. *Arch Intern Med.* 2003;163(13):1566-1572. DOI: 10.1001/archinte.163.13.1566.

72. West J, Logan RFA, Smith CJ, Hubbard RB, Card TR. Malignancy and mortality in people with coeliac disease: population based cohort study. *BMJ.* 2004;329(7468):716. DOI: 10.1136/bmj.38169.486701.7C.

73. Solaymani-Dodaran M, West J, Logan RF. Long-term mortality in people with celiac disease diagnosed in childhood compared with adulthood: a population-based cohort study. *Am J Gastroenterol.* 2007;102(4):864-870. DOI: 10.1111/j.1572-0241.2007.01111.x.
74. Ludvigsson JF, Montgomery SM, Ekbom A, Brandt L, Granath F. Small-intestinal histopathology and mortality risk in celiac disease. *Jama.* 2009;302(11):1171-1178. DOI: 10.1001/jama.2009.1320.
75. Grainge MJ, West J, Card TR, Holmes GK. Causes of death in people with celiac disease spanning the pre- and post-serology era: a population-based cohort study from Derby, UK. *Am J Gastroenterol.* 2011;106(5):933-939. DOI: 10.1038/ajg.2010.506.
76. Abdul Sultan A, Crooks CJ, Card T, Tata LJ, Fleming KM, West J. Causes of death in people with coeliac disease in England compared with the general population: a competing risk analysis. *Gut.* 2015;64(8):1220-1226. DOI: 10.1136/gutjnl-2014-308285.
77. Holmes GKT, Muirhead A. Mortality in coeliac disease: a population-based cohort study from a single centre in Southern Derbyshire, UK. *BMJ Open Gastroenterol.* 2018;5(1):e000201-e000201. DOI: 10.1136/bmjgast-2018-000201.
78. Quarpong W, Card TR, West J, Solaymani-Dodaran M, Logan RF, Grainge MJ. Mortality in people with coeliac disease: Long-term follow-up from a Scottish cohort. *United European Gastroenterol J.* 2019;7(3):377-387. DOI: 10.1177/2050640618814662.
79. Lebwohl B, Green PHR, Söderling J, Roelstraete B, Ludvigsson JF. Association Between Celiac Disease and Mortality Risk in a Swedish Population. *Jama.* 2020;323(13):1277-1285. DOI: 10.1001/jama.2020.1943.
80. Koskinen I, Virta LJ, Huhtala H, Ilus T, Kaukinen K, Collin P. Overall and Cause-Specific Mortality in Adult Celiac Disease and Dermatitis Herpetiformis Diagnosed in the 21st Century. *Am J Gastroenterol.* 2020. DOI: 10.14309/ajg.0000000000000665.
81. Ludvigsson JF, West J, Card T, Appelros P. Risk of stroke in 28,000 patients with celiac disease: a nationwide cohort study in Sweden. *J Stroke Cerebrovasc Dis.* 2012;21(8):860-867. DOI: 10.1016/j.jstrokecerebrovasdis.2011.05.008.

82. Wei L, Spiers E, Reynolds N, Walsh S, Fahey T, MacDonald TM. The association between coeliac disease and cardiovascular disease. *Aliment Pharmacol Ther.* 2008;27(6):514-519. DOI: 10.1111/j.1365-2036.2007.03594.x.

83. Gajulapalli RD, Pattanshetty DJ. Risk of coronary artery disease in celiac disease population. *Saudi J Gastroenterol.* 2017;23(4):253-258. DOI: 10.4103/sjg.SJG_616_16.

84. Miehsler W, Reinisch W, Valic E, et al. Is inflammatory bowel disease an independent and disease specific risk factor for thromboembolism? *Gut.* 2004;53(4):542-548. DOI: 10.1136/gut.2003.025411.

85. Ludvigsson JF, Welander A, Lassila R, Ekbom A, Montgomery SM. Risk of thromboembolism in 14,000 individuals with coeliac disease. *Br J Haematol.* 2007;139(1):121-127. DOI: 10.1111/j.1365-2141.2007.06766.x.

86. Ramagopalan SV, Wotton CJ, Handel AE, Yeates D, Goldacre MJ. Risk of venous thromboembolism in people admitted to hospital with selected immune-mediated diseases: record-linkage study. *BMC Med.* 2011;9:1-1. DOI: 10.1186/1741-7015-9-1.

87. Zöller B, Li X, Sundquist J, Sundquist K. Risk of pulmonary embolism in patients with autoimmune disorders: a nationwide follow-up study from Sweden. *Lancet (London, England).* 2012;379(9812):244-249. DOI: 10.1016/s0140-6736(11)61306-8.

88. Johannesdottir SA, Erichsen R, Horváth-Puhó E, Schmidt M, Sørensen HT. Coeliac disease and risk of venous thromboembolism: a nationwide population-based case-control study. *Br J Haematol.* 2012;157(4):499-501. DOI: 10.1111/j.1365-2141.2012.09030.x.

89. Bagot CN, Arya R. Virchow and his triad: a question of attribution. *Br J Haematol.* 2008;143(2):180-190. DOI: 10.1111/j.1365-2141.2008.07323.x.

90. Hallert C, Grant C, Grehn S, et al. Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Aliment Pharmacol Ther.* 2002;16(7):1333-1339. DOI: 10.1046/j.1365-2036.2002.01283.x.

91. Dickey W, Ward M, Whittle CR, et al. Homocysteine and related B-vitamin status in coeliac disease: Effects of gluten exclusion and histological recovery. *Scand J Gastroenterol.* 2008;43(6):682-688. DOI: 10.1080/00365520701881118.

92. Hoss GRW, Poloni S, Blom HJ, Schwartz IVD. Three Main Causes of Homocystinuria: CBS, cblC and MTHFR Deficiency. What do they Have in

Common? *J Inborn Errors Metab Screen.* 2019;7. DOI: 10.1590/2326-4594-jiems-2019-0007.

93. Wilcox GM, Mattia AR. Celiac sprue, hyperhomocysteinemia, and MTHFR gene variants. *J Clin Gastroenterol.* 2006;40(7):596-601. DOI: 10.1097/00004836-200608000-00007.

94. Hadithi M, Mulder CJ, Stam F, et al. Effect of B vitamin supplementation on plasma homocysteine levels in celiac disease. *World J Gastroenterol.* 2009;15(8):955-960. DOI: 10.3748/wjg.15.955.

95. Djuric D, Jakovljevic V, Zivkovic V, Srejovic I. Homocysteine and homocysteine-related compounds: an overview of the roles in the pathology of the cardiovascular and nervous systems. *Can J Physiol Pharmacol.* 2018;96(10):991-1003. DOI: 10.1139/cjpp-2018-0112.

96. Bauer KA. Hypercoagulability--a new cofactor in the protein C anticoagulant pathway. *N Engl J Med.* 1994;330(8):566-567. DOI: 10.1056/nejm199402243300810.

97. Boucelma M, Saadi M, Boukrara H, Bensalah D, Hakem D, Berrah A. Association of celiac disease and cerebral venous thrombosis: report of two cases. *J Mal Vasc.* 2013;38(1):47-51. DOI: 10.1016/j.jmv.2012.11.003.

98. Berthoux E, Fabien N, Chayvialle JA, Ninet J, Durieu I. Adult celiac disease with thrombosis: a case series of seven patients. Role of thrombophilic factors. *Rev Med Interne.* 2011;32(10):600-604. DOI: 10.1016/j.revmed.2011.02.025.

99. Kallel L, Matri S, Karoui S, Fekih M, Boubaker J, Filali A. Deep venous thrombosis related to protein S deficiency revealing celiac disease. *Am J Gastroenterol.* 2009;104(1):256-257. DOI: 10.1038/ajg.2008.48.

100. Maclean PS, Tait RC. Hereditary and acquired antithrombin deficiency: epidemiology, pathogenesis and treatment options. *Drugs.* 2007;67(10):1429-1440. DOI: 10.2165/00003495-200767100-00005.

101. Moretti S, Mrakic-Sposta S, Roncoroni L, et al. Oxidative stress as a biomarker for monitoring treated celiac disease. *Clin Transl Gastroenterol.* 2018;9(6):157-157. DOI: 10.1038/s41424-018-0031-6.

102. Comba A, Çaltepe G, Yank K, Gör U, Yüce Ö, Kalayc AG. Assessment of Endothelial Dysfunction With Adhesion Molecules in Patients With Celiac Disease. *J Pediatr Gastroenterol Nutr.* 2016;63(2):247-252. DOI: 10.1097/mpg.0000000000001138.

103. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol.* 2007;7(10):803-815. DOI: 10.1038/nri2171.
104. De Marchi S, Chiarioni G, Prior M, Arosio E. Young adults with coeliac disease may be at increased risk of early atherosclerosis. *Aliment Pharmacol Ther.* 2013;38(2):162-169. DOI: 10.1111/apt.12360.
105. Coleman NS, Foley S, Dunlop SP, et al. Abnormalities of serotonin metabolism and their relation to symptoms in untreated celiac disease. *Clin Gastroenterol Hepatol.* 2006;4(7):874-881. DOI: 10.1016/j.cgh.2006.04.017.
106. Aksu K, Donmez A, Keser G. Inflammation-induced thrombosis: mechanisms, disease associations and management. *Curr Pharm Des.* 2012;18(11):1478-1493. DOI: 10.2174/138161212799504731.
107. Sjöber K, Eriksson S, Tenngart B, Roth EB, Leffler H, Stenberg P. Factor XIII and tissue transglutaminase antibodies in coeliac and inflammatory bowel disease. *Autoimmunity.* 2002;35(5):357-364. DOI: 10.1080/73-0891693021000005402.
108. Laine O, Pitkänen K, Lindfors K, et al. Elevated serum antiphospholipid antibodies in adults with celiac disease. *Dig Liver Dis.* 2018;50(5):457-461. DOI: 10.1016/j.dld.2017.11.018.
109. Denham JM, Hill ID. Celiac Disease and Autoimmunity: Review and Controversies. *Curr Allergy Asthma Rep.* 2013;13(4):347-353. DOI: 10.1007/s11882-013-0352-1.
110. Pitocco D, Giubilato S, Martini F, et al. Combined atherogenic effects of celiac disease and type 1 diabetes mellitus. *Atherosclerosis.* 2011;217(2):531-535. DOI: 10.1016/j.atherosclerosis.2011.04.042.
111. Mormile R. Celiac disease and atherosclerosis: An immunologic puzzle to be solved? *Immunol Lett.* 2016;180:75-76. DOI: 10.1016/j.imlet.2016.10.003.
112. Bijl M. Endothelial activation, endothelial dysfunction and premature atherosclerosis in systemic autoimmune diseases. *Neth J Med.* 2003;61(9):273-277. DOI:
113. Mager DR, Qiao J, Turner J. Vitamin D and K status influences bone mineral density and bone accrual in children and adolescents with celiac disease. *Eur J Clin Nutr.* 2012;66(4):488-495. DOI: 10.1038/ejcn.2011.176.
114. Korkmaz H, Sozen M, Kebapcilar L. Increased arterial stiffness and its relationship with inflammation, insulin, and insulin resistance in celiac disease.

Eur J Gastroenterol Hepatol. 2015;27(10):1193-1199. DOI: 10.1097/meg.0000000000000437.

115. Baskurt OK, Meiselman HJ. Blood rheology and haemodynamics. *Semin Thromb Haemost.* 2003;29(5):435-450. DOI: 10.1055/s-2003-44551.
116. Késmárky G, Kenyeres P, Rábai M, Tóth K. Plasma viscosity: a forgotten variable. *Clin Haemorheol Microcirc.* 2008;39(1-4):243-246. DOI: 10.3233/CH-2008-1088.
117. Pretorius E. Erythrocyte deformability and eryptosis during inflammation, and impaired blood rheology. *Clin Haemorheol Microcirc.* 2018;69(4):545-550. DOI: 10.3233/ch-189205.
118. Santos MJ, Pedro LM, Canhão H, et al. Haemorheological parameters are related to subclinical atherosclerosis in systemic lupus erythematosus and rheumatoid arthritis patients. *Atherosclerosis.* 2011;219(2):821-826. DOI: 10.1016/j.atherosclerosis.2011.08.026.
119. Luquita A, Urli L, Svetaz MJ, et al. Erythrocyte aggregation in rheumatoid arthritis: Cell and plasma factor's role. *Clin Haemorheol Microcirc.* 2009;41(1):49-56. DOI: 10.3233/CH-2009-1154.
120. Papp J, Sandor B, Toth A, et al. Altered microrheological parameters in Raynaud's phenomenon. *Clin Haemorheol Microcirc.* 2017;65(1):23-29. DOI: 10.3233/ch-162069.
121. Vayá A, Todolí J, Calvo J, Romagnoli M, Ricart JM. Haemorheological profile in patients with systemic sclerosis. *Clin Haemorheol Microcirc.* 2008;40(3):243-248. DOI: 10.3233/CH-2008-1133.
122. Akman T, Akarsu M, Akpinar H, Resmi H, Taylan E. Erythrocyte deformability and oxidative stress in inflammatory bowel disease. *Dig Dis Sci.* 2012;57(2):458-464. DOI: 10.1007/s10620-011-1882-9.
123. Maharshak N, Arbel Y, Shapira I, et al. Increased strength of erythrocyte aggregates in blood of patients with inflammatory bowel disease. *Inflamm Bowel Dis.* 2009;15(5):707-713. DOI: 10.1002/ibd.20838.
124. Novacek G, Vogelsang H, Genser D, et al. Changes in blood rheology caused by Crohn's disease. *Eur J Gastroenterol Hepatol.* 1996;8(11):1089-1093. DOI: 10.1097/00042737-199611000-00011.

125. Zilberman L, Rogowski O, Rozenblat M, et al. Inflammation-related erythrocyte aggregation in patients with inflammatory bowel disease. *Dig Dis Sci.* 2005;50(4):677-683. DOI: 10.1007/s10620-005-2556-2.
126. Lobo AJ, Jones SC, Juby LD, Axon AT. Plasma viscosity in inflammatory bowel disease. *J Clin Pathol.* 1992;45(1):54-57. DOI: 10.1136/jcp.45.1.54.
127. Yakar T, Cosar AM, Gokturk HS, et al. Plasma viscosity: a potential predictor of both medical treatment response and clinical stage of ulcerative colitis. *Ann Ital Chir.* 2016;87:531-543. DOI:
128. Vandenbroucke JP, von Elm E, Altman DG, et al. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. *PLoS Med.* 2007;4(10):e297-e297. DOI: 10.1371/journal.pmed.0040297.
129. 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-e026315. DOI: 10.1136/bmjopen-2018-026315.
130. Goldhaber SZ. Risk factors for venous thromboembolism. *J Am Coll Cardiol.* 2010;56(1):1-7. DOI: 10.1016/j.jacc.2010.01.057.
131. Previtali E, Bucciarelli P, Passamonti SM, Martinelli I. Risk factors for venous and arterial thrombosis. *Blood Transfus.* 2011;9(2):120-138. DOI: 10.2450/2010.0066-10.
132. Svedlund J, Sjödin I, Dotevall G. GSRS--a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. *Dig Dis Sci.* 1988;33(2):129-134. DOI: 10.1007/bf01535722.
133. Lansink-Hartgring AO, Hoffmann R, van den Bergh W, de Vries A. Changes in Red Blood Cell Properties and Platelet Function during Extracorporeal Membrane Oxygenation. *J Clin Med.* 2020;9(4):1168. DOI: 10.3390/jcm9041168.
134. Baskurt OK, Boynard M, Cokelet GC, et al. New guidelines for haemorheological laboratory techniques. *Clin Haemorheol Microcirc.* 2009;42(2):75-97. DOI: 10.3233/CH-2009-1202.
135. Di Sabatino A, Rosado MM, Cazzola P, et al. Splenic hypofunction and the spectrum of autoimmune and malignant complications in celiac disease. *Clin Gastroenterol Hepatol.* 2006;4(2):179-186. DOI: 10.1016/s1542-3565(05)00982-1.

136. William BM, Corazza GR. Hyposplenism: a comprehensive review. Part I: basic concepts and causes. *Hematology.* 2007;12(1):1-13. DOI: 10.1080/10245330600938422.
137. Corazza GR, Frisoni M, Vaira D, Gasbarrini G. Effect of gluten-free diet on splenic hypofunction of adult coeliac disease. *Gut.* 1983;24(3):228-230. DOI: 10.1136/gut.24.3.228.
138. Sategna Guidetti C, Scaglione N, Martini S. Red cell distribution width as a marker of coeliac disease: a prospective study. *Eur J Gastroenterol Hepatol.* 2002;14(2):177-181. DOI: 10.1097/00042737-200202000-00012.
139. Patel KV, Mohanty JG, Kanapuru B, Hesdorffer C, Ershler WB, Rifkind JM. Association of the red cell distribution width with red blood cell deformability. *Adv Exp Med Biol.* 2013;765:211-216. DOI: 10.1007/978-1-4614-4989-8_29.
140. Gyawali P, Richards RS, Uba Nwose E. Erythrocyte morphology in metabolic syndrome. *Expert Rev Hematol.* 2012;5(5):523-531. DOI: 10.1586/ehm.12.47.
141. Radosinska J, Vrbjar N. The role of red blood cell deformability and Na,K-ATPase function in selected risk factors of cardiovascular diseases in humans: focus on hypertension, diabetes mellitus and hypercholesterolemia. *Physiol Res.* 2016;65 Suppl 1:S43-54. DOI: 10.33549/physiolres.933402.
142. Diaz-Castro J, Muriel-Neyra C, Martin-Masot R, Moreno-Fernandez J, Maldonado J, Nestares T. Oxidative stress, DNA stability and evoked inflammatory signaling in young celiac patients consuming a gluten-free diet. *Eur J Nutr.* 2019. DOI: 10.1007/s00394-019-02013-5.
143. Vayá A, Rivera L, de la Espriella R, et al. Red blood cell distribution width and erythrocyte deformability in patients with acute myocardial infarction. *Clin Haemorheol Microcirc.* 2015;59(2):107-114. DOI: 10.3233/ch-131751.
144. Caimi G, Valenti A, Presti R. Acute myocardial infarction in young adults: evaluation of the haemorheological pattern at the initial stage, after 3 and 12 months. *Annali dell'Istituto superiore di sanita.* 2007;43(2):139. DOI: not available.
145. Marton Z, Horvath B, Alexy T, et al. Follow-up of haemorheological parameters and platelet aggregation in patients with acute coronary syndromes. *Clin Haemorheol Microcirc.* 2003;29(2):81-94. DOI: not available.

146. Vayá A, López JM, Contreras MT, et al. Erythrocyte deformability in survivors of acute myocardial infarction measured by two different methodologies. 2002;27(1):17-25. DOI: not available.
147. Wasilewski J, Turczyński B, Słowińska L, Kowalik V, Osadnik T, Poloński L. Haemorheological factors and myocardial reperfusion in patients with ST-elevation myocardial infarction undergoing primary coronary intervention. *Kardiol Pol.* 2007;65(7):778-785. DOI: not available.
148. Sargento L, Saldanha C, Monteiro J, Perdigão C, e Silva JM. Long-term prognostic value of protein C activity, erythrocyte aggregation and membrane fluidity in transmural myocardial infarction. *Thromb Haemost.* 2005;94(2):380-388. DOI: 10.1160/th04-12-0791.
149. Szikszai Z, Fekete I, Imre S. A comparative study of haemorheological parameters in transient ischemic attack and acute ischemic stroke patients: possible predictive value. *Clin Haemorheol Microcirc.* 2003;28(1):51-57. DOI: not available.
150. Banerjee R, Nageswari K, Puniyani R. Association of haemorheological parameters and risk of stroke in hypertensives of Indian origin. *Clin Exp Hypertens.* 2000;22(7-8):687-694. DOI: not available.
151. Capuano P, Catalano G, Garruti G, et al. The effects of weight loss due to gastric banding and lifestyle modification on red blood cell aggregation and deformability in severe obese subjects. *Int J Obes (Lond).* 2012;36(3):342-347. DOI: 10.1038/ijo.2011.94.
152. Wiewiora M, Piecuch J, Glûck M, Slowinska-Lozynska L, Sosada K. The impacts of super obesity versus morbid obesity on red blood cell aggregation and deformability among patients qualified for bariatric surgery. *Clin Haemorheol Microcirc.* 2014;58(4):543-550. DOI: 10.3233/ch-141814.
153. Vayá A, Cámara R, Hernádez-Mijares A, et al. Erythrocyte deformability in morbid obesity before bariatric surgery. Influence of abdominal obesity. *Clin Haemorheol Microcirc.* 2010;46(4):313-320. DOI: 10.3233/ch-2010-1357.
154. Solá E, Vayá A, Santaolaria ML, et al. Erythrocyte deformability in obesity measured by ektacytometric techniques. *Clin Haemorheol Microcirc.* 2007;37(3):219-227. DOI: not available.
155. Miossec P, Zkhiri F, Pariès J, David-Dufilho M, Devynck MA, Valensi PE. Effect of pravastatin on erythrocyte rheological and biochemical properties in poorly

controlled Type 2 diabetic patients. *Diabet Med.* 1999;16(5):424-430. DOI: 10.1046/j.1464-5491.1999.00083.x.

156. Saklamaz A, Comlekci A, Temiz A, et al. The beneficial effects of lipid-lowering drugs beyond lipid-lowering effects: a comparative study with pravastatin, atorvastatin, and fenofibrate in patients with type IIa and type IIb hyperlipidemia. *Metabolism.* 2005;54(5):677-681. DOI: 10.1016/j.metabol.2004.12.012.

157. Szapary L, Horvath B, Marton Z, et al. Short-term effect of low-dose atorvastatin on haemorrheological parameters, platelet aggregation and endothelial function in patients with cerebrovascular disease and hyperlipidaemia. *CNS Drugs.* 2004;18(3):165-172. DOI: 10.2165/00023210-200418030-00003.

158. Linde T, Sandhagen B, Hägg A, Mörlin C, Danielson BG. Decreased blood viscosity and serum levels of erythropoietin after anti-hypertensive treatment with amlodipine or metoprolol: results of a cross-over study. *J Hum Hypertens.* 1996;10(3):199-205. DOI: not available.

159. Forst T, Weber MM, Löbig M, et al. Pioglitazone in addition to metformin improves erythrocyte deformability in patients with Type 2 diabetes mellitus. *Clin Sci (Lond).* 2010;119(8):345-351. DOI: 10.1042/cs20100161.

160. Magyar K, Halmosi R, Palfi A, et al. Cardioprotection by resveratrol: A human clinical trial in patients with stable coronary artery disease. *Clin Haemorheol Microcirc.* 2012;50(3):179-187. DOI: 10.3233/CH-2011-1424.

161. Bakker N, Schoorl M, Stoutjesdijk E, Houdijk APJ. Erythrocyte deformability and aggregability in patients undergoing colon cancer surgery and effects of two infusions with omega-3 fatty acids. *Clin Haemorheol Microcirc.* 2020;74(3):287-297. DOI: 10.3233/ch-190687.

162. Radosinska J, Horvathova M, Frimmel K, et al. Acute dark chocolate ingestion is beneficial for haemodynamics via enhancement of erythrocyte deformability in healthy humans. *Nutr Res.* 2017;39:69-75. DOI: 10.1016/j.nutres.2017.03.002.

163. Weng X, Roederer GO, Beaulieu R, Cloutier G. Contribution of acute-phase proteins and cardiovascular risk factors to erythrocyte aggregation in normolipidemic and hyperlipidemic individuals. *Thromb Haemost.* 1998;80(6):903-908. DOI: not available.

164. Pfafferott C, Moessmer G, Ehrly AM, Bauersachs RM. Involvement of erythrocyte aggregation and erythrocyte resistance to flow in acute coronary syndromes. *Clin Haemorheol Microcirc.* 1999;21(1):35-43. DOI: not available.

165. Demiroğlu H, Barişta I, Dündar S. Erythrocyte aggregability in patients with coronary heart disease. *Clin Haemorheol Microcirc.* 1996;16(3):313-317. DOI: not available.
166. Lakshmi AB, Uma P, Venkatachalam C, Nageswar Rao GS. A simple slide test to assess erythrocyte aggregation in acute ST-elevated myocardial infarction and acute ischemic stroke: its prognostic significance. *Indian J Pathol Microbiol.* 2011;54(1):63-69. DOI: 10.4103/0377-4929.77327.
167. Shapira I, Rotstein R, Fusman R, et al. Combined leukocyte and erythrocyte aggregation in patients with acute myocardial infarction. *Int J Cardiol.* 2001;78(3):299-305. DOI: not available.
168. Steinvil A, Berliner S, Shapira I, et al. Time to rheology in acute myocardial infarction: Inflammation and erythrocyte aggregation as a consequence and not necessarily as precursors of the disease. *Clin Res Cardiol.* 2010;99:651-656. DOI: 10.1007/s00392-010-0167-1.
169. Kesmarky G, Toth K, Habon L, Vajda G, Juricskay I. Haemorheological parameters in coronary artery disease. *Clin Haemorheol Microcirc.* 1998;18(4):245-251. DOI: not available.
170. Tzoulaki I, Murray GD, Lee AJ, Rumley A, Lowe GD, Fowkes FG. Relative value of inflammatory, haemostatic, and rheological factors for incident myocardial infarction and stroke: the Edinburgh Artery Study. *Circulation.* 2007;115(16):2119-2127. DOI: 10.1161/circulationaha.106.635029.
171. Koenig W, Sund M, Filipiak B, Döring A, Löwel H, Ernst E. Plasma viscosity and the risk of coronary heart disease: results from the MONICA-Augsburg Cohort Study, 1984 to 1992. *Arterioscler Thromb Vasc Biol.* 1998;18(5):768-772. DOI: 10.1161/01.atv.18.5.768.
172. Lowe G, Rumley A, Norrie J, et al. Blood rheology, cardiovascular risk factors, and cardiovascular disease: the West of Scotland Coronary Prevention Study. *Thromb Haemost.* 2000;84(4):553-558. DOI: not available.
173. Ekizler FA, Cay S, Tak BT, et al. Usefulness of the whole blood viscosity to predict stent thrombosis in ST-elevation myocardial infarction. *Biomark Med.* 2019;13(15):1307-1320. DOI: 10.2217/bmm-2019-0246.
174. Cetin EH, Cetin MS, Canpolat U, et al. Prognostic significance of whole blood viscosity estimated by de Simone's formula in ST-elevation myocardial infarction. *Biomark Med.* 2016;10(5):495-511. DOI: 10.2217/bmm.16.10.

175. Boekholdt SM, Kramer MH. Arterial thrombosis and the role of thrombophilia. *Semin Thromb Haemost.* 2007;33(6):588-596. DOI: 10.1055/s-2007-985755.
176. Sacco RL, Anand K, Lee HS, et al. Homocysteine and the risk of ischemic stroke in a triethnic cohort: the NOrthern MAnhattan Study. *Stroke.* 2004;35(10):2263-2269. DOI: 10.1161/01.Str.0000142374.33919.92.
177. Woodward M, Rumley A, Rumley A, et al. The association between homocysteine and myocardial infarction is independent of age, sex, blood pressure, cholesterol, smoking and markers of inflammation: the Glasgow Myocardial Infarction Study. *Blood Coagul Fibrinolysis.* 2006;17(1):1-5. DOI: 10.1097/01.mbc.0000195919.71950.f7.
178. Zanini B, Mazzoncini E, Lanzarotto F, et al. Impact of gluten-free diet on cardiovascular risk factors. A retrospective analysis in a large cohort of coeliac patients. *Dig Liver Dis.* 2013;45(10):810-815. DOI: 10.1016/j.dld.2013.04.001.

12. Scientometrics

Scientific papers:

- Total: 49
- English-language: 47

Impact factor (up to 9th Oct 2020 based on MTMT2):

- First and last author: 33.797
- Cummulative: 160.721

Citations (up to 9th Oct 2020 based on MTMT2):

- Independent: 163
- Cummulative: 185
- Hirsh index: 7

List of publications

Papers upon which this thesis relies (n=2, cummulative 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. DOI: 10.1136/bmjopen-2018-026315 (**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 (article in press). DOI: not available yet (**Q1, IF: 3.968**).

Papers closely 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. DOI: 10.1371/journal.pone.0212329 (**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. DOI: 10.1111/iji.12415 (**Q3, IF: 1.130**).
3. Bajor J, **Szakács Z**, Vincze Á. Response to Letter to the Editor: Relevance of HLA-DQB1*02 allele in predisposing to coeliac disease. *Int J Immunogenet.* 2019;46(4):276-277. DOI: 10.1111/iji.12428 (**Q3, IF: 1.130**).
4. **Szakács Z**, Gede N, Gyöngyi Z, et al. A Call for Research on the Prognostic Role of Follow-Up Histology in Celiac Disease: A Systematic Review. *Front Physiol.* 2019;10:1408. DOI: 10.3389/fphys.2019.01408 (**Q2, IF: 3.367**).

5. **Szakács Z**, Mátrai P, Hegyi P, et al. Younger age at diagnosis predisposes to mucosal recovery in celiac disease on a gluten-free diet: A meta-analysis. *PLoS One.* 2017;12(11):e0187526. DOI: 10.1371/journal.pone.0187526 (**Q1, IF: 2.766**).

Other papers (n=41):

1. Bocskai T, Kovács M, **Szakács Z**, et al. Is the bispectral index monitoring protective against postoperative cognitive decline? A systematic review with meta-analysis. *PLoS One.* 2020;15(2):e0229018. DOI: 10.1371/journal.pone.0229018 (**Q1, IF: 2.740**).
2. Czumbel LM, Kiss S, Farkas N, …**Szakács Z**, …et al. Saliva as a Candidate for COVID-19 Diagnostic Testing: A Meta-Analysis. *Front Med.* 2020;7:465. DOI: 10.3389/fmed.2020.00465 (**Q1, IF: 3.900**).
3. Bui TQ, Bui QVP, Németh D, …**Szakács Z**, …et al. Epidermal Growth Factor is Effective in the Treatment of Diabetic Foot Ulcers: Meta-Analysis and Systematic Review. *Int J Environ Res Public Health.* 2019;16(14). DOI: 10.3390/ijerph16142584 (**Q2, IF: 2.849**).
4. Demcsák A, Lantos T, Bálint ER, …**Szakács Z**,… et al. PPIs Are Not Responsible for Elevating Cardiovascular Risk in Patients on Clopidogrel - A Systematic Review and Meta-Analysis. *Front Physiol.* 2018;9:1550. DOI: 10.3389/fphys.2018.01550 (**Q2, IF: 3.201**).
5. Dunás-Varga V, Hegyi P, Izbéki F, **Szakács Z**, Varjú P, Gajdán L. Drug induced acute pancreatitis. *Cent Eur J Gastroenterol Hepatol.* 2019;5(3):142-144. DOI: 10.33570/CEUJGH.5.2.142 (**not listed, IF: 0**).
6. Eitmann S, Németh D, Hegyi P, …**Szakács Z**,… et al. Maternal overnutrition impairs offspring's insulin sensitivity: A systematic review and meta-analysis. *Matern Child Nutr.* 2020:e13031. DOI: 10.1111/mcn.13031 (**Q1, IF: 2.789**).
7. Erős A, Soós A, Hegyi P, …**Szakács Z**,… et al. Sarcopenia as an independent predictor of the surgical outcomes of patients with inflammatory bowel disease: a meta-analysis. *Surg Today.* 2019. DOI: 10.1007/s00595-019-01893-8 (**Q1, IF: 1.878**).
8. Erős A, Soós A, Hegyi P, …**Szakács Z**,… et al. Spotlight on Transition in Patients With Inflammatory Bowel Disease: A Systematic Review. *Inflamm Bowel Dis.* 2020;26(3):331-346. DOI: 10.1093/ibd/izz173 (**Q1, IF: 4.261**).

9. Erős A, Veres G, Tárnok A, …**Szakács Z**,… et al. A cross-sectional survey on the transitional care of adolescents with inflammatory bowel disease in Hungary. *J Ped Nurs.* 2020 (article in press). DOI: 10.1016/j.pedn.2020.06.002 (**not listed, IF: 1.495**).
10. Erőss B, Molnár Z, **Szakács Z**, … et al. Personalised health education against health damage of COVID-19 epidemic in the elderly Hungarian population (PROACTIVE-19): protocol of an adaptive randomised controlled clinical trial. *Trials.* 2020;21:809. DOI: 10.1186/s13063-020-04733-0 **(Q1, IF: 1.883)**.
11. Fábián A, Bor R, Gede N, …**Szakács Z**,… et al. Double Stenting for Malignant Biliary and Duodenal Obstruction: A Systematic Review and Meta-Analysis. *Clin Transl Gastroenterol.* 2020;11(4):e00161. DOI: 10.14309/ctg.0000000000000161 (**Q1, IF: 3.968**).
12. Farkas N, Hanák L, Mikó A, …**Szakács Z**,… et al. A Multicenter, International Cohort Analysis of 1435 Cases to Support Clinical Trial Design in Acute Pancreatitis. *Front Physiol.* 2019;10:1092. DOI: 10.3389/fphys.2019.01092 (**Q2, IF: 3.367**).
13. Földi M, Farkas N, Kiss S, …**Szakács Z**,… et al. Obesity is a risk factor for developing critical condition in COVID-19 patients: A systematic review and meta-analysis. *Obes Rev.* 2020 (article in press). DOI: 10.1111/obr.13095 (**Q1, IF: 7.310**).
14. Garami A, Shimansky YP, Rumbus Z, …**Szakács Z**,… et al. Hyperthermia induced by transient receptor potential vanilloid-1 (TRPV1) antagonists in human clinical trials: Insights from mathematical modeling and meta-analysis. *Pharmacol Ther.* 2020;208:107474. DOI: 10.1016/j.pharmthera.2020.107474 (**Q1, IF: 10.557**).
15. Gombos K, Herczeg R, Erőss B, …**Szakács Z**,… et al. Translating Scientific Knowledge to Government Decision Makers Has Crucial Importance in the Management of the COVID-19 Pandemic. *Popul Health Manag.* 2020 (article in press). DOI: 10.1089/pop.2020.0159 (**Q1, IF: 2.138**).
16. Hágendorn R, Farkas N, Vincze Á, …**Szakács Z**,… et al. Chronic kidney disease severely deteriorates the outcome of gastrointestinal bleeding: A meta-analysis. *World J Gastroenterol.* 2017;23(47):8415-8425. DOI: 10.3748/wjg.v23.i47.8415 (**Q1, IF: 3.300**).

17. Hegyi P, Petersen OH, Holgate S, …**Szakács Z**,… et al. Academia Europaea Position Paper on Translational Medicine: The Cycle Model for Translating Scientific Results into Community Benefits. *J Clin Med.* 2020;9(5). DOI: 10.3390/jcm9051532 (**not listed, IF: 3.303**).
18. Hegyi P*, **Szakács Z***, Sahin-Tóth M. Lipotoxicity and Cytokine Storm in Severe Acute Pancreatitis and COVID-19. *Gastroenterology.* 2020:S0016-5085(0020)34938-34936. DOI: 10.1053/j.gastro.2020.07.014 (**Q1, IF: 17.373**).
19. Horváth T, Hanák L, Hegyi P, …**Szakács Z**,… et al. Hydroxyapatite-coated implants provide better fixation in total knee arthroplasty. A meta-analysis of randomized controlled trials. *PLoS One.* 2020;15(5):e0232378. DOI: 10.1371/journal.pone.0232378 (**Q1, IF: 2.740**).
20. Kerémi B, Márta K, Farkas K, …**Szakács Z**,… et al. Effects of Chlorine Dioxide on Oral Hygiene - A Systematic Review and Meta-analysis. *Curr Pharm Des.* 2020;26(25):3015-3025. DOI: 10.2174/1381612826666200515134450 (**Q2, IF: 2.208**).
21. Kiss Z, Tél B, Farkas N, …**Szakács Z**,… et al. Eosinophil Counts in the Small Intestine and Colon of Children Without Apparent Gastrointestinal Disease: A Meta-analysis. *J Ped Nurs.* 2018;67(1):6-12. DOI: 10.1097/mpg.0000000000001904 (**not listed, IF: 1.563**).
22. Kocsis T, Molnár B, Németh D, …**Szakács Z**,… et al. Probiotics have beneficial metabolic effects in patients with type 2 diabetes mellitus: a meta-analysis of randomized clinical trials. *Sci Rep.* 2020;10(1):11787. DOI: 10.1038/s41598-020-68440-1 (**Q1, IF: 3.998**).
23. Koukkoullis A, Tóth I, Gede N, …**Szakács Z**,… et al. Endoscopic versus microscopic stapes surgery outcomes: A meta-analysis and systematic review. *Laryngoscope.* 2020;130(8):2019-2027. DOI: 10.1002/lary.28353 (**Q1, IF: 2.465**).
24. Kupó P, **Szakács Z**, Solymár M, et al. Direct Anticoagulants and Risk of Myocardial Infarction, a Multiple Treatment Network Meta-Analysis. *Angiology.* 2020;71(1):27-37. DOI: 10.1177/0003319719874255 (**Q2, IF: 2.255**).
25. Merész G, Szabó S, Dóczy V, Hölgyesi Á, **Szakács Z**. Relative frequency of urinary tract infections in patients affected by diabetes mellitus type 2 treated with metformin and SGLT2 inhibitor. Network meta-analysis. *Orv Hetil.* 2020;161(13):491-501. DOI: 10.1556/650.2020.31690 (**Q3, IF: 0.497**).

26. Mosztbacher D, Hanák L, Farkas N, …**Szakács Z**,… et al. Hypertriglyceridemia-induced acute pancreatitis: A prospective, multicenter, international cohort analysis of 716 acute pancreatitis cases. *Pancreatology.* 2020;20(4):608-616. DOI: 10.1016/j.pan.2020.03.018 (**Q1, IF: 3.629**).
27. Németh B, Murányi E, Hegyi P, …**Szakács Z**,… et al. Asymmetric dimethylarginine levels in preeclampsia - Systematic review and meta-analysis. *Placenta.* 2018;69:57-63. DOI: 10.1016/j.placenta.2018.07.010 (**Q1, IF: 2.773**).
28. Otto C, Tárnok A, Erős A, …**Szakács Z**,… et al. Planned Transition of Adolescent Patients with Inflammatory Bowel Disease Results in Higher Remission Rates. *J Ped Nurs.* 2019;45:62-66. DOI: 10.1016/j.pedn.2019.02.003 (**not listed, IF: 1.495**).
29. Pap I, Tóth I, Gede N, …**Szakács Z**,… et al. Endoscopic type I tympanoplasty is as effective as microscopic type I tympanoplasty but less invasive - A meta-analysis. *Clin otolaryngol.* 2019;44(6):942-953. DOI: 10.1111/coa.13407 (**Q1, IF: 2.197**).
30. Párniczky A, Lantos T, Tóth EM, …**Szakács Z**,… et al. Antibiotic therapy in acute pancreatitis: From global overuse to evidence based recommendations. *Pancreatology.* 2019;19(4):488-499. DOI: 10.1016/j.pan.2019.04.003 (**Q1, IF: 3.629**).
31. Pécsi D, Farkas N, Hegyi P, …**Szakács Z**,… et al. Transpancreatic Sphincterotomy Is Effective and Safe in Expert Hands on the Short Term. *Dig Dis Sci.* 2019;64(9):2429-2444. DOI: 10.1007/s10620-019-05640-4 (**Q2, IF: 3.570**).
32. **Szakács Z**, Erőss B, Soós A, et al. Baveno Criteria Safely Identify Patients With Compensated Advanced Chronic Liver Disease Who Can Avoid Variceal Screening Endoscopy: A Diagnostic Test Accuracy Meta-Analysis. *Front Physiol.* 2019;10:1028. DOI: 10.3389/fphys.2019.01028 (**Q2, IF: 3.367**).
33. **Szakács Z**, Faluhelyi N, Fincsur A, et al. Acute appendicitis in a patient with perianal Crohn's disease receiving infliximab. *Orv Hetil.* 2018;159(10):405-409. DOI: 10.1556/650.2018.30982 (**Q3, IF: 0.564**).
34. **Szakács Z**, Gede N, Pécsi D, et al. Aging and Comorbidities in Acute Pancreatitis II.: A Cohort-Analysis of 1203 Prospectively Collected Cases. *Front Physiol.* 2018;9:1776. DOI: 10.3389/fphys.2018.01776 (**Q2, IF: 3.201**).
35. Szakó L, Mátrai P, Hegyi P, …**Szakács Z**,… et al. Endoscopic and surgical drainage for pancreatic fluid collections are better than percutaneous drainage:

Meta-analysis. *Pancreatology.* 2020;20(1):132-141. DOI: 10.1016/j.pan.2019.10.006 (**Q1, IF: 3.629**).

36. Szemes K, Soós A, Hegyi P, …**Szakács Z**,… et al. Comparable Long-Term Outcomes of Cyclosporine and Infliximab in Patients With Steroid-Refractory Acute Severe Ulcerative Colitis: A Meta-Analysis. *Front Med.* 2019;6:338. DOI: 10.3389/fmed.2019.00338 (**Q1, IF: 3.900**).
37. Tél B, Stubnya B, Gede N, …**Szakács Z**,… et al. Inflammatory bowel diseases elevate the risk of developing acute pancreatitis. *Pancreas*. 2020 (article in press). DOI: not available yet (**Q1, IF: 2.920**).
38. Tinusz B, Soós A, Hegyi P, …**Szakács Z**,… et al. Efficacy and safety of stenting and additional oncological treatment versus stenting alone in unresectable esophageal cancer: A meta-analysis and systematic review. *Radiother Oncol.* 2020;147:169-177. DOI: 10.1016/j.radonc.2020.05.015 (**Q1, IF: 4.856**).
39. Tinusz B, Szapáry L, Paládi B, …**Szakács Z**,… et al. Short-Course Antibiotic Treatment Is Not Inferior to a Long-Course One in Acute Cholangitis: A Systematic Review. *Dig Dis Sci.* 2019;64(2):307-315. DOI: 10.1007/s10620-018-5327-6 (**Q2, IF: 3.570**).
40. Tóth B, Hegyi P, Lantos T, …**Szakács Z**,… et al. The Efficacy of Saffron in the Treatment of Mild to Moderate Depression: A Meta-analysis. *Plant Med.* 2019;85(1):24-31. DOI: 10.1055/a-0660-9565 (**Q1, IF: 2.687**).
41. Váncsa S, Németh D, Hegyi P, …**Szakács Z**,… et al. Fatty Liver Disease and Non-Alcoholic Fatty Liver Disease Worsen the Outcome in Acute Pancreatitis: A Systematic Review and Meta-Analysis. *J Clin Med.* 2020;9(9):2698. DOI: 10.3390/jcm9092698 (**not listed, IF: 3.303**).
42. Varjú P, Gede N, **Szakács Z**, et al. Lactose intolerance but not lactose maldigestion is more frequent in patients with irritable bowel syndrome than in healthy controls: A meta-analysis. *Neurogastroenterol Motil.* 2019;31(5):e13527. DOI: 10.1111/nmo.13527 (**Q1, IF: 2.946**).

13. Appendix

Appendix 1. Comorbidities and regular medications of study participants

Subject identifier	Comorbid conditions	Medications
	Coeliac patients	
HER001	Raynaud syndrome, sicca syndrome	Pantoprazole
HER002	Hypertension, hypothyroidism, hyperprolactinaemia, iron deficiency, metrorrhagia, infertility, Turner syndrome (mosaic), retrocelebellar cyst	Iron replacement therapy, levothyroxine, carvedilol, bromocriptine, hesperidin-diosmin
HER003	Hypertension, dermatitis herpetiformis	Dapsone
HER004	Bronchial asthma, allergy, osteopaenia	Desloratadine, budesonide-formoterol inhaler
HER005	Hypertension	None
HER006	Dermatitis herpetiformis, infertility	None
HER008	Osteopaenia, infertility	None
HER012	Hypertension, sarcoidosis, glaucoma, seborrhoea capitis, dermatitis herpetiformis	Telmisartan, norethisterone
HER013	Hypertension, hypothyroidism, osteoporosis, dermatitis herpetiformis	Perindopril, levothyroxine, zolpidem
HER014	Bronchial asthma, benign prostate hyperplasia, type 2 diabetes mellitus	Ciclesonide, tamsulosin, dutasteride
HER015	Osteopaenia	None
HER016	Primary biliary cholangitis, osteoporosis	Ursodeoxycholic acid, pantoprazole
HER017	None	None
HER018	Strabism, hereditary hearing loss	None
HER019	Hypertension, anxiety, osteopaenia, hypothyroidism, allergic rhinitis	Moxonidine, amlodipine, duloxetine, levothyroxine
HER020	Thoracic outlet syndrome, endometriosis, preeclampsia	Iron replacement therapy
HER021	Oligoarthritis (atypical), alopecia areata, osteoporosis, corrected ventricular septal defect	None
HER022	Osteopaenia, dysmenorrhea	None
HER024	None	None
HER025	Hypertension, NAFLD	Valsartan
HER026	None	None
HER029	Anaemia, hyperthyroidism	None
HER031	Airway allergy, dysmenorrhea	None
HER032	None	None
HER033	GERD, dermatitis herpetiformis	Omeprazole
HER034	GERD	Esomeprazole
HER037	None	None
HER038	Hypertension, GERD, osteoporosis	Lisinopril, famotidine
HER039	Hypertension, osteoporosis	Telmisartan, nebivolol
HER040	None	None
HER041	Benign tumour of the spleen and the small bowel, osteoporosis	Betahistine, alprazolam, vinpocetine, nicergoline, pantoprazole

Appendix 1 (continued)

Subject identifier	Comorbid conditions	Medications
HER042	Metal allergy	None
HER043	Food and pollen allergy	None
HER044	Sjögren's syndrome, autoimmune hepatitis and primary biliary cholangitis overlap	None
HER045	Hypertension, osteopaenia	Perindopril, naproxen, allopurinol
HER046	Pollen allergy	None
HER047	Hypertension, dermatitis herpetiformis, acute myocardial infarction (old)	Clonazepam, carvedilol, perindopril, indapamide, escitalopram, clopidogrel, mirtazapine, montelucast, formoterol-beclomethasone inhaler
HER048	None	None
HER050	None	Nebivolol
HER051	Bronchial asthma, lactose intolerance	Budesonide-formoterol inhaler
HER053	Hypertension, small-fibre neuropathy	adjuvant analgesics (unknown)
HER054	None	None
HER055	None	None
HER057	Psoriasis, lichen simplex, type 2 diabetes mellitus	Insulin, iron replacement therapy
HER058	None	None
HER059	None	None
HER060	None	None
HER061	Thyroid adenoma (post-stumectomy), cataract, benign prostate hyperplasia, type 2 diabetes mellitus	Alfuzosin, antidiabetic therapy (unknown)
HER062	Hypertension	Perindopril, amlodipine
HER063	None	Antihistamine (unknown)
	Control subjects	
HER201	GERD, infertility, cervix carcinoma (operated on it >5 years age, no relapse)	Rabeprazole
HER202	Hypertension, GERD	Valsartan
HER203	GERD	None
HER205	Hypertension	Nebivolol
HER206	Anxiety, GERD, small intestinal bacterial overgrowth (cured)	Bisoprolol, duloxetine, alprazolam
HER207	None	None
HER208	Graves's disease	Methotyrine
HER209	Pollen allergy	Desloratadine
HER210	None	None
HER211	None	None
HER212	None	None
HER213	None	None

Appendix 1 (continued)

Subject identifier	Comorbid conditions	Medications
HER214	Hypertension, bronchial asthma, chronic kidney disease (mild with nearly normal creatinine), age-related macula degeneration, benign prostate hyperplasia	Allopurinol, atorvastatin, budesonide-formoterol inhaler, pantoprazole, perindopril
HER215	Hypertension	Bisoprolol, clopamide
HER218	Migraine	None
HER219	GERD	Lansoprazole
HER220	None	Bisoprolol
HER221	Psoriasis	None
HER222	Hypertension, glaucoma, diverticulosis	Valsartan
HER223	Chronic pancreatitis, hypertension, Hashimoto-thyroiditis, bronchial asthma, rheumatoid arthritis, pulmonary embolism (old)	Valsartan, hydrochlorothiazide, levothyroxine, allopurinol, budesonide-formoterol inhaler
HER224	Hashimoto-thyroiditis, occlusive peripheral artery disease, hypertension, type 2 diabetes mellitus	Famotidine, duloxetine, levothyroxine, irbesartan, bisoprolol, simvastatin, folic acid, alprazolam, insulin analogue antidiabetic drug (unknown)
HER225	Hypertension, preeclampsia	Nebivolol, perindopril
HER226	Adenomatous colon polyp, hypertension, type 2 diabetes mellitus	Bisoprolol, rosuvastatin, tioctanic acid, pantoprazole, telmisartan
HER228	None	None
HER230	Lactose intolerance	None
HER232	Wilson’s disease	Penicillamine
HER233	Bronchial asthma, hypertension, type 2 diabetes mellitus	Metformin, montelucast, ciclesonid inhaler, ramipril, amlodipine, nebivolol
HER234	None	None
HER239	Cryptogenic cirrhosis (Child A)	None
HER240	None	None
HER241	None	None
HER242	Hypertension	None
HER244	NAFLD	None
HER245	None	None
HER246	Airway allergy	None
HER247	None	None
HER248	Hypertension	Losartan
HER249	None	None
HER250	None	None
HER253	Hashimoto-thyroiditis	Levothyroxine
HER254	Allergic rhinitis	Desloratadine
HER255	Psoriasis, lactose intolerance	None
HER257	None	None
HER258	None	None

Appendix 1 (continued)

Subject identifier	Comorbid conditions	Medications
HER260	Depression, migraine, goitre (non-functioning), GERD	Alprazolam, sertraline, desloratadine, iron replacement therapy
HER262	Acne vulgaris	None
HER264	Lactose intolerance, airway allergy	None
HER270	None	None
HER272	None	None
HER273	None	None

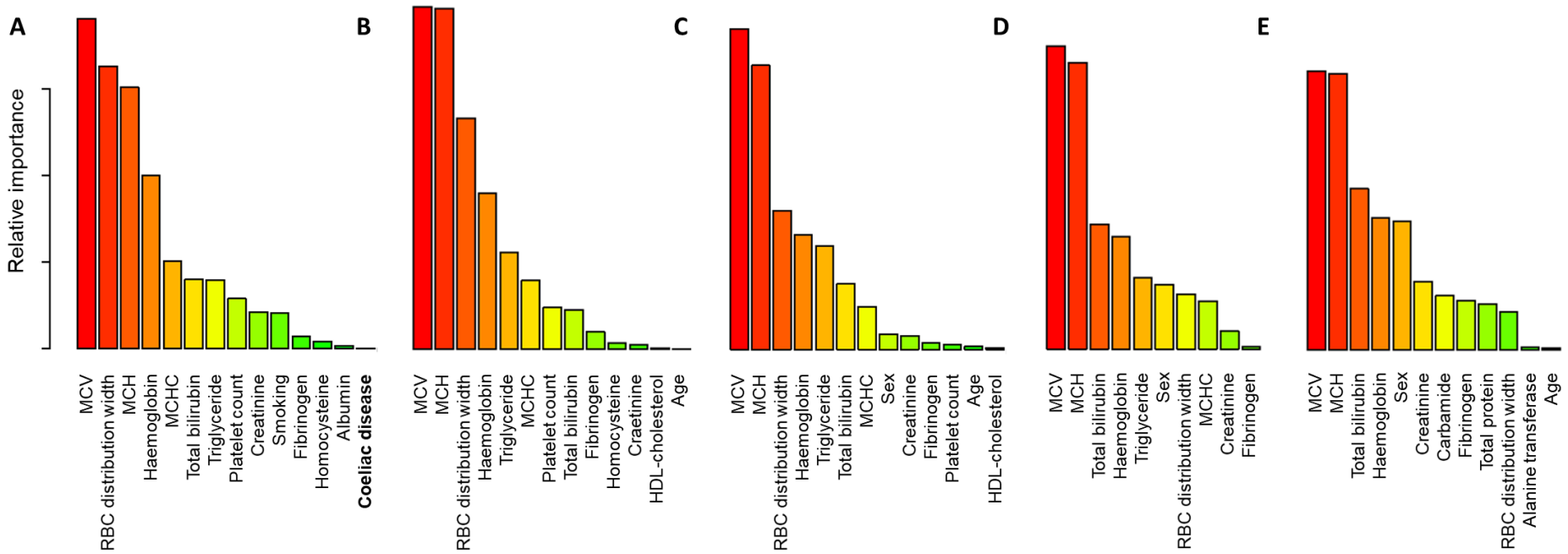
The subject identifiers were given before screening for eligibility; therefore, they are not necessarily consecutive. GERD: gastro-oesophageal reflux disease; NAFLD: non-alcoholic fatty liver disease.

Appendix 2. Assessment of symptoms per the Gastrointestinal Symptoms Rating Scale

	Coeliac group (n=50)	Control group (n=50)	p-value
Overall score	1.63; 1.50 [1.00–3.13]	1.69; 1.40 [1.00–4.27]	0.790
Items			
Pain or discomfort in the upper abdomen	1.30; 1.00 [1.00–5.00]	1.46; 1.00 [1.00–5.00]	0.411
Heartburn	1.54; 1.00 [1.00–7.00]	1.82; 1.00 [1.00–5.00]	**0.041**
Acid reflux	1.34; 1.00 [1.00–5.00]	1.74; 1.00 [1.00–6.00]	0.094
Hunger pains	1.88; 1.00 [1.00–6.00]	1.70; 1.00 [1.00–6.00]	0.322
Nausea	1.44; 1.00 [1.00–7.00]	1.56; 1.00 [1.00–7.00]	0.935
Rumbling	1.68; 1.00 [1.00–5.00]	1.92; 1.00 [1.00–5.00]	0.361
Bloating	2.28; 1.50 [1.00–6.00]	2.18; 1.00 [1.00–6.00]	0.743
Burping	1.30; 1.00 [1.00–6.00]	1.28; 1.00 [1.00–5.00]	0.582
Flatulence	1.52; 1.00 [1.00–5.00]	1.76; 1.00 [1.00–6.00]	0.540
Constipation	1.80; 1.00 [1.00–7.00]	1.70; 1.00 [1.00–6.00]	0.759
Diarrhea	1.48; 1.00 [1.00–6.00]	1.58; 1.00 [1.00–6.00]	0.729
Loose stools	2.06; 1.00 [1.00–7.00]	1.84; 1.00 [1.00–6.00]	0.383
Hard stools	2.02; 1.00 [1.00–7.00]	1.80; 1.00 [1.00–5.00]	1.000
Urgent need to have a bowel movement	1.32; 1.00 [1.00–5.00]	1.44; 1.00 [1.00–5.00]	0.885
Sensation of not completely emptying the bowels	1.50; 1.00 [1.00–5.00]	1.58; 1.00 [1.00–6.00]	0.429

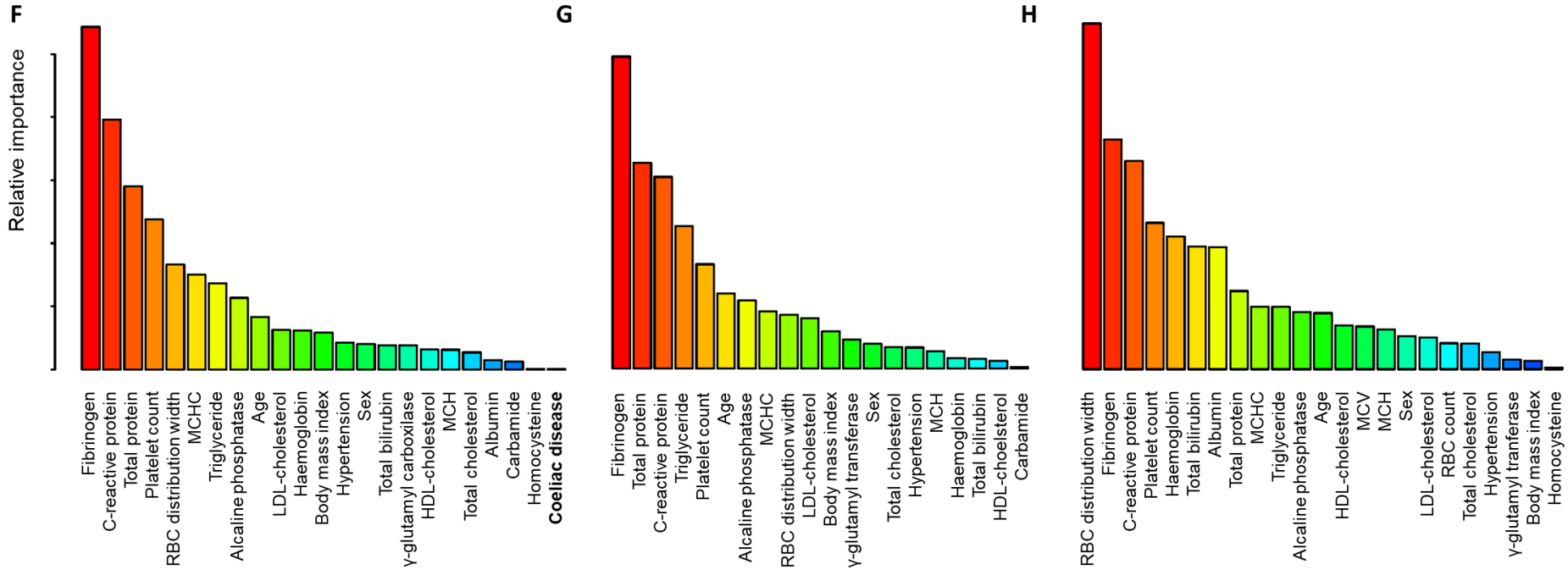
Boldface type indicates a statistically significant difference. Values are given in mean; median [min–max] format. P-values were generated with the Mann-Whitney test.

Appendix 3. Important predictors of erythrocyte deformability



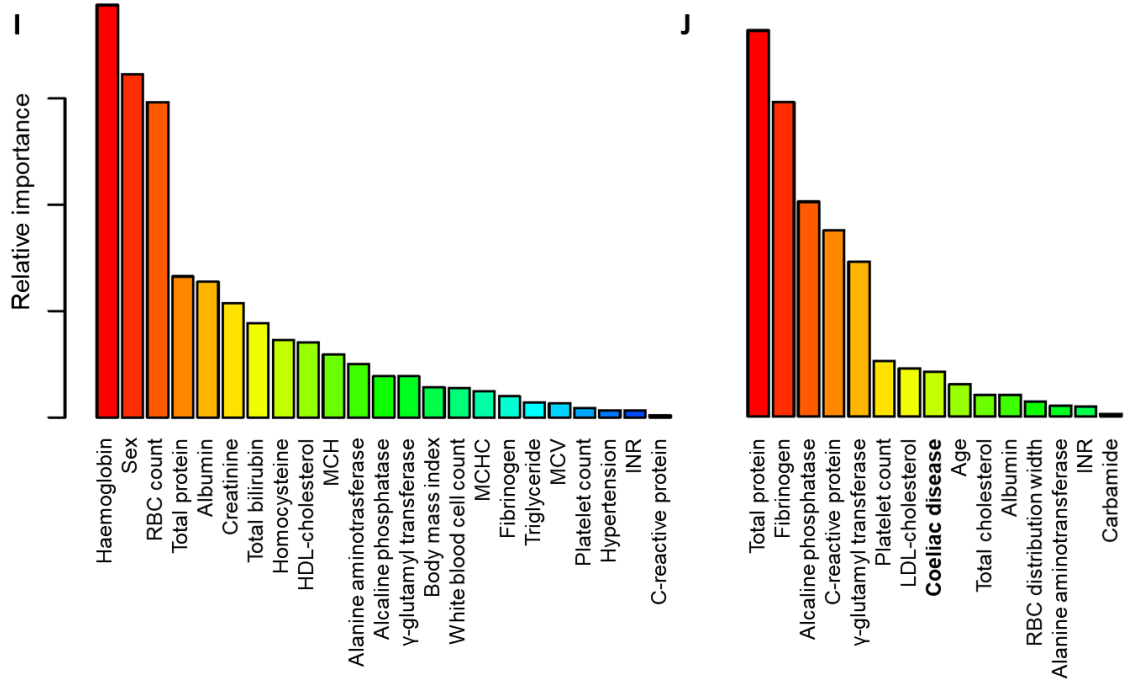
Panels A, B, C, D and E show the elongation indices at 3, 1.69, 0.95, 0.53 and 0.3 Pa, respectively. The figures were generated with random forest analysis. We added 34 co-variates to the model, but only the important predictors of the outcome are shown. The relative importance is proportional to the height of the bars. The number of participants is 97 in the analysis. CeD: coeliac disease; HDL: high-density lipoprotein; INR: international normalized ratio; 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.

Appendix 3 (continued). Important predictors of erythrocyte deformability



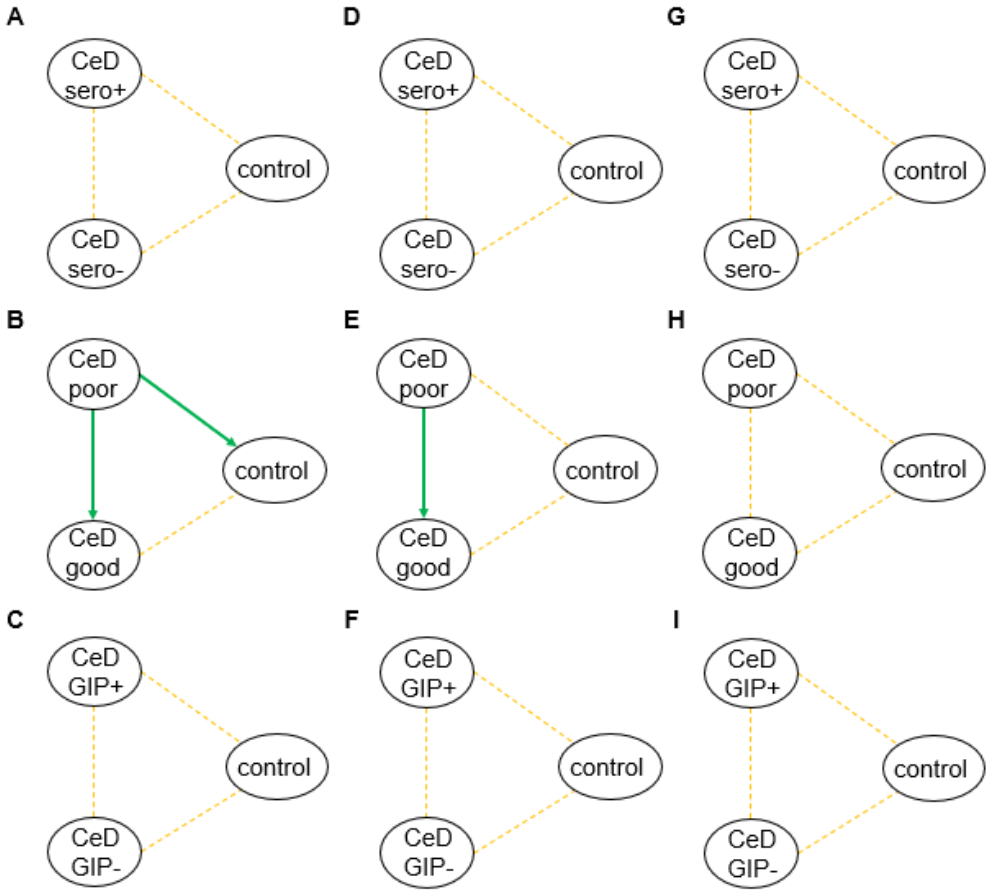
Panels F, G and H show the aggregation index (%), aggregation half-time (sec) and threshold shear rate (1/sec), respectively. The figures were generated with random forest analysis. We added 34 co-variates to the model, but only the important predictors of the outcome are shown. The relative importance is proportional to the height of the bars. The number of participants is 97 in the analysis. CeD: coeliac disease; HDL: high-density lipoprotein; INR: international normalized ratio; 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.

Appendix 3 (continued). Important predictors of erythrocyte deformability



Panel I and H show the whole blood viscosity and plasma viscosity, 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. CeD: coeliac disease, HDL: high-density lipoprotein, INR: international normalized ratio, LDL: low-density lipoprotein, MCH: mean corpuscular haemoglobin, MCHC mean corpuscular haemoglobin concentration; MCV mean corpuscular volume, RBC: red blood cell.

Appendix 4. Model for the association of erythrocyte aggregation, whole blood viscosity and plasma viscosity with dietary adherence



A, **D and G**: coeliac-specific serology and erythrocyte aggregation, whole blood viscosity and plasma viscosity, respectively. **B**, **E and H**: dietary review through interview and erythrocyte aggregation, whole blood viscosity and plasma viscosity, respectively. **C**, **F and I**: urine-GIP detection and erythrocyte aggregation, whole blood viscosity and plasma viscosity, 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. Yellow dashed lines indicate no significant difference between groups. CeD: coeliac disease, GIP: gluten immunogenic peptide. The figure is the author's own work.

Diet-dependent and Diet-independent Hemorheological Alterations in Celiac Disease: A Case-Control Study

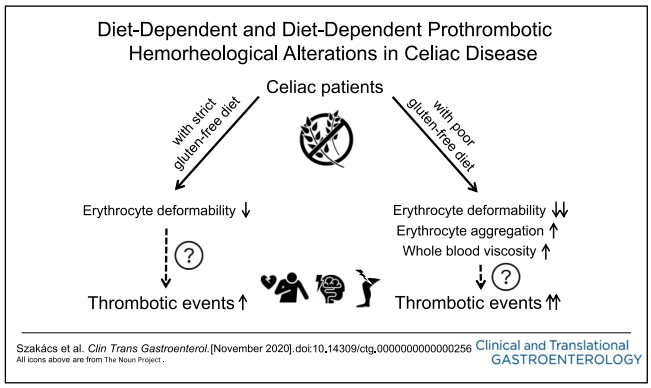
Zsolt Szakács, MD[1,2], Beáta Csiszár, MD[2,3], Mátyás Nagy[1], Nelli Farkas, PhD[1,4], Péter Kenyeres, MD, PhD[2,3], Adrienn Erős, MD, PhD[1,5], Alizadeh Hussain, MD, PhD[2,6], Katalin Márta, MD, PhD[1,2], Andrea Szentesi, PhD[1], Margit Tőkés-Füzesi, MD, PhD[7], Tímea Berki, MD, PhD, ScD[8], Áron Vincze, MD, PhD[2,9], Kálmán Tóth, MD, PhD, ScD[2,3], Péter Hegyi, MD, PhD, ScD[1,2] and Judit Bajor, MD, PhD[9]

INTRODUCTION: **Hemorheology is the study of the flow properties of the blood and its elements, which, together with natural anticoagulants, are important determinants of cardiovascular events. This study aimed to assess hemorheological and natural anticoagulant profiles of patients with celiac disease (CeD) comprehensively.**

METHODS: **Our study is a case-control study (registered under ISRCTN49677481) comparing patients with CeD with age- and sex-matched control subjects (1:1). We measured erythrocyte deformability (ED) at high (3–30 Pa) and low shears (0.3–3 Pa), erythrocyte aggregation, whole blood viscosity, plasma viscosity, and natural anticoagulants (protein C, protein S, and antithrombin activity). Adherence to gluten-free diet was estimated through dietary interview and urine gluten immunogenic peptide (urine GIP) detection.**

RESULTS: **After matching, we analyzed the data of 100 study participants. ED at high shears was impaired in CeD ($P < 0.05$ for all shears, confirmed by random forest analysis) independently of findings on CeD-specific serological assessment and urine GIP detection but slightly dependently on dietary adherence ($P = 0.025$ for 30 Pa shear). ED at low shears seemed to be impaired only in urine GIP+ CeD patients ($P < 0.05$ for all comparisons with urine GIP− CeD patients and control subjects). All parameters describing erythrocyte aggregation and whole blood viscosity were shifted toward a prothrombotic direction in patients with CeD with poor dietary adherence compared with those with good dietary adherence. Plasma viscosity and activity of natural anticoagulants did not differ across groups.**

DISCUSSION: **We observed diet-dependent and diet-independent prothrombotic hemorheological alterations in CeD, which can contribute to the elevated cardiovascular risk. The untoward metabolic changes during gluten-free diet, which can further aggravate hemorheological status, may indicate the implementation of prevention strategies.**



[1]Institute for Translational Medicine, Medical School, University of Pécs, Pécs, Hungary; [2]János Szentágothai Research Center, University of Pécs, Pécs, Hungary; [3]Division of Cardiology, First Department of Medicine, Medical School, University of Pécs, Pécs, Hungary; [4]Institute of Bioanalysis, Medical School, University of Pécs, Pécs, Hungary; [5]Heim Pál National Insititute of Pediatrics, Budapest, Hungary; [6]Division of Hematology, First Department of Medicine, Medical School, University of Pécs, Pécs, Hungary; [7]Department of Laboratory Medicine, Medical School, University of Pécs, Pécs, Hungary; [8]Department of Immunology and Biotechnology, Medical School, University of Pécs, Pécs, Hungary; [9]Division of Gastroenterology, First Department of Medicine, Medical School, University of Pécs, Pécs, Hungary. **Correspondence:** Judit Bajor, MD, PhD. E-mail: bajor.judit@pte.hu.

Received June 11, 2020; accepted September 16, 2020; published online XXXX

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Clinical and Translational Gastroenterology 2020;00:e00256. https://doi.org/10.14309/ctg.0000000000000256

INTRODUCTION

Celiac disease (CeD) is a chronic, immune-mediated disorder, which develops on gluten exposure in genetically susceptible individuals (1). CeD affects about 1% of the population; nevertheless, its prevalence is still increasing (2).

The disease carries the risk of severe complications including but not restricted to nutritional deficiencies, metabolic bone disease, and malignant tumors (1). Besides, cardiovascular (CV) comorbidities should be highlighted. A meta-analysis (2012) of population-based studies suggested an increased 1.19-fold risk of CV-related deaths for patients with CeD (3), confirmed by a recent (2020) population-based Swedish cohort study (4). The increased CV risk can be explained with several pathophysiological mechanisms. Prothrombotic alterations include malabsorption (vitamin K deficiency with subsequent protein C and protein S deficiency, or vitamin B_{12}/B_9 deficiency with subsequent hyperhomocysteinemia), thrombophilic antibodies, accelerated atherosclerosis, endothelial and platelet dysfunction, comorbid conditions (e.g., antiphospholipid syndrome), subclinical chronic inflammation, and genetics (5,6). Energy density and nutrient composition of gluten-free diet (GFD) often deviate from the optimal (7–10), raising concerns about a variety of modifiable CV risk factors to change unfavorably (11).

Besides the factors mentioned above, the hemorheological profile should be taken into account when potential prothrombotic alterations are investigated. Hemorheology (in other words, blood rheology) is the study of the flow properties of the blood and its elements. Hemorheological indicators, such as hematocrit (HTC), whole blood viscosity (WBV), plasma viscosity (PV), erythrocyte deformability (ED), and erythrocyte aggregation (EA), play fundamental roles in the maintenance of microcirculation (12–14). Besides, partly through increased endothelial shear stress (15), an altered hemorheological profile has long been known to be an essential determinant of thrombogenesis (16,17) and was reported in various immune-mediated diseases including systemic lupus erythematosus (18,19) and rheumatoid arthritis (19,20). Hemorheological indicators are extensively studied in inflammatory bowel disease; reports indicated (sometimes, activity dependent) alterations of ED, EA, fibrinogen, and PV in both Crohn's disease and ulcerative colitis (21–27). Nevertheless, little (or rather no) attention has been paid to hemorheology in CeD. Similarly, natural anticoagulants (protein C, protein S, and antithrombin), the physiological inhibitors of the coagulation cascade, and thrombogenesis were never investigated in patients with CeD in a comparative study.

Our aim was the comprehensive evaluation of hemorheological and natural anticoagulant profiles of patients with CeD with particular focus on the effects of dietary adherence to explore alterations which can contribute to the elevated CV risk.

METHODS

The study was approved by the Regional and Local Research Ethics Committee (University of Pécs, Pécs, Hungary; Ref No. 6917) and registered in the ISRCTN Registry under registration number ISRCTN49677481. Full technical details of the protocol are published elsewhere (28). This report follows the STROBE Statement (29).

Design, setting, and eligibility

Our study is a single-center observational study with a case-control design. Adults (aged ≥18 years) were recruited consecutively from the gastroenterology outpatient clinic of our

Table 1. Hemorheology-related terms and measurements

Parameter (abbreviation)	Measurement (abbreviation or symbol, unit)	Definition	Unfavorable alteration[a]
Erythrocyte deformability (ED)	Elongation index (EI, no unit)	Change in the shape of red blood cells at high (from EI_{30Pa} to EI_{3Pa} in this study) and low shear stresses (from EI_{3Pa} to $EI_{0.3Pa}$ in this study) (shown in Figure 1 inlet)	↓
Erythrocyte aggregation (EA)	Aggregation index (AI, %)	Integral in the change of light intensity 10 s after disaggregation	↑
	Aggregation half-time ($t_{1/2}$, s)	The time required for achieving half of the maximal aggregation after disaggregation	↓
	Threshold shear rate (γ, s^{-1})	Lowest shear that can maintain complete disaggregation	↑
Viscosity	Whole blood viscosity (WBV, mPa·s)	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	↑
	Plasma viscosity (PV, mPa·s)		↑

[a]Regarding thrombus formation. AI, $t_{1/2}$, and γ were measured with Laser-assisted Optical Rotational Cell Analyzer (LORCA; R&R Mechatronics, Hoorn, the Netherlands), EI was measured with laser-diffraction ektacytometry with a LORCA, and WBV and PV were measured with Brookfield DV-III Ultra LV Programmable rotational viscometer (Brookfield Engineering Labs, Middleboro, MA) at mid-shear ($90\ s^{-1}$).

academic hospital between June 2017 and May 2018. Cases were patients with biopsy-confirmed CeD diagnosed according to the current guidelines (30,31). Control subjects were individuals in whom CeD was excluded by the treating gastroenterologist specialist (30,31). Exclusion criteria from the study are detailed in the prestudy protocol (28).

Outcomes

Outcomes included hemorheological parameters (describing HTC, WBV, PV, fibrinogen, ED, and EA) and the activity of natural anticoagulants (antithrombin, protein C, and protein S).

Flow and timing

Potential participants with a definitive diagnosis were screened for eligibility by a gastroenterologist specialist. If consented to participate, detailed medical history was obtained, and medical files were revised; then, the questionnaires were administered by the same person. The visit ended with a dietary interview, followed by blood and urine collection in our central laboratory unit.

Questionnaires, laboratory measurements, and dietary interview

We used a thrombophilia questionnaire to assess arterial and venous thrombotic risk factors (for the items of the questionnaire, see Supplementary Digital Content 1, http://links.lww.com/CTG/A418) and the Gastrointestinal Symptom Rating Scale to assess the severity of gastrointestinal symptoms (32).

Laboratory tests were performed from venous blood as detailed in the prestudy protocol (28). Hemorheology-related terms and measurements are detailed in Table 1. We adhered to the guidelines proposed by the International Expert Panel for Standardization of Hemorheological Methods during the tests (33).

Urine gluten immunogenic peptides (urine GIPs) were measured by a point-of-care test according to the test's user guide (iVYCHECK GIP Urine, Biomedal, Spain).

In patients with CeD, dietary adherence was estimated through a dietary interview conducted by an accredited dietitian who judged adherence on a visual analog scale between 1 (regular gluten-containing diet) and 10 (perfect GFD). The same person ensured that control subjects did not follow a GFD.

Subgroup analysis

Patients with CeD were divided by CeD-specific serology (based on tissue transglutaminase antibody [tTG]-IgA/IgG and endomysial antibody [EMA] IgA/IgG levels with the cutoff from the test's user guide), by estimated dietary adherence based on dietary interview (scores <8 points were chosen to indicate poor dietary adherence), and by the results of urine GIP measurement (interpreted according to the test's user guide). CeD-specific serology rather reflects the intensity of the ongoing immune response and is sensitive to detect major dietary transgressions, whereas urine GIP positivity rather reflects gluten intake of the past 2–3 days (34).

Sample size and data analysis

Because hemorheological indicators were never determined in CeD, we planned to recruit 100 age- and sex-matched participants (1:1 ratio) in the first phase to determine further target numbers for the second phase (28). Completing the first phase, we realized that to reach the level of significance for the observed mean differences between patients with CeD and control subjects at $\alpha = 0.05$ and $\beta = 0.80$, we should recruit an unfeasible number of subjects (>1,000 patients for EA indicators, 247 patients for PV, and 289 patients for WBV per group), so that we decided to stop the study for futility.

Table 2. Clinical characteristics of the study population

	Celiac group (n = 50)	Control group (n = 50)
Age (mean; median [min–max], yr)	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)
Thrombotic events in first-degree relatives (n, %)	14 (28.0)	12 (24.0)
Current smoker (n, %)	9 (18.0)	6 (12.0)
Alcohol consumption (≥7 units/wk) (n, %)	3 (6.0)	4 (8.0)
Chronic alcohol abuse (n, %)	3 (6.0)	2 (4.0)
Body mass index (mean; median [min–max], 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)
Lower limb varicose veins (n, %)	14 (28.0)	11 (22.0)
Surgery (≤1 yr) (n, %)	7 (14.0)	7 (14.0)
Immobilization (≤14 d) (n, %)	1 (2.0)	0 (0.0)
Travel by plane, bus, or car (≥6 hr continuously; ≤14 d) (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, $P \geq 0.05$ for all comparisons otherwise.

After matching by age (±5 years tolerance) and sex (±10% tolerance), descriptive statistics were performed. Categorical variables were given in proportions (% of total). Continuous variables were given with central tendencies (mean and/or median) and measure of dispersion (SD, quartiles, and/or range) based on distribution determined by the visual inspection of Q-Q plots. Logarithmic transformation was applied to normalize the distribution in the case of $t_{1/2}$, γ, WBV, PV, antithrombin, and protein S activity.

In univariate analysis, we used the Welch, Mann-Whitney, χ^2, and Fisher tests; one-way ANOVA (with the Tukey *post hoc* test), Kruskal-Wallis test (with the Mann-Whitney *post hoc* test) and Bonferonni correction (where appropriate)). In multivariate analysis, we used the random forest method to determine and graphically display the relative importance of each predictor. During prediction, 100 random forests with 500 trees in each were grown using conditional inference method to avoid bias toward dependent predictors and overfitting (35).

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

Table 3. Biochemical characteristics of the study population

	Celiac 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[a]
Creatinine (umol/L)	71.6; 68.5 (39.0–110.0)	72.8; 68.5 (49.0–105.0)	0.668
Carbamide (mmol/L)	4.2; 4.1 (2.2–7.3)	4.5; 4.3 (2.2–7.9)	0.257
Total bilirubin (umol/L)	8.1; 7.9 (2.7–20.0)	9.5; 8.1 (2.1–24.5)	0.120[a]
AST (U/L)	25.6; 19.0 (10.0–200.0)	22.0; 20.0 (10.0–89.0)	0.806[a]
ALT (U/L)	24.1; 18.0 (9.0–158.0)	21.7; 18.0 (8.0–60.0)	0.901[a]
ALP (U/L)	74.3; 68.0 (36.0–269.0)	67.9; 67.5 (35.0–108.0)	0.696[a]
γ-GT (U/L)	23.1; 16.0 (10.0–94.0)	26.1; 16.0 (7.0–210.0)	0.825[a]
Total protein (g/L)[b]	74.5; 74.7 (62.4–93.0)	75.3; 74.7 (67.7–85.2)	0.445
Albumin (g/L)	48.2; 47.8 (37.8–56.6)	49.2; 49.1 (43.9–57.8)	0.182
Ultrasensitive CRP (mg/L)	3.4; 1.8 (0.0–23.5)	2.3; 1.4 (0.0–10.1)	0.342[a]
ESR (mm/hr)	9.0; 4.0 (1.0–46.0)	6.1; 5.0 (1.0–27.0)	0.808[a]
Prothrombin time (s)	11.4; 11.3 (9.6–14.0)	11.2; 11.2 (9.8–13.2)	0.252
Thrombin time (s)	14.5; 14.6 (12.6–16.9)	14.3; 14.2 (12.0–17.4)	0.213
APTI (s)	28.6; 28.3 (23.3–36.1)	30.1; 28.7 (19.0–72.9)	0.176
INR	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 (%)	59; 59 (41–83)	58; 58 (43–74)	0.353
Neutrophil granulocytes (G/L)	4.4; 4.2 (1.7–13.4)	3.9; 3.6 (1.8–9.2)	0.134
Lymphocytes (%)	30; 29 (9–45)	32; 33 (16–47)	0.171
Lymphocytes (G/L)	2.1; 2.0 (1.2–4.2)	2.1; 2.1 (1.1–3.9)	0.947
Monocytes (%)	7.3; 7.0 (2.6–11.5)	7.4; 7.4 (4.2–10.7)	0.773
Monocytes (G/L)	0.52; 0.48 (0.15–1.14)	0.50; 0.47 (0.18–0.99)	0.469
Eosinophil granulocytes (%)	2.5; 2.0 (0.3–8.5)	1.8; 1.5 (0.0–7.8)	**0.023**
Eosinophil granulocytes (G/L)	0.18; 0.14 (0.01–0.60)	0.11; 0.10 (0.00–0.47)	**0.003**
Basophil granulocytes (%)	0.77; 0.60 (0.30–4.00)	0.70; 0.70 (0.20–1.90)	0.487
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
Hemoglobin (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

Table 3. (*continued*)

	Celiac group (n = 50)	Control group (n = 50)	*P* value
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
Homocysteine (μmol/L)	9.0; 8.3 (5.1–13.7)	8.7; 7.7 (4.4–42.9)	**0.040**[a]
Vitamin B_{12} (ng/L)	450; 450 (156–785)	396; 424 (192–613)	0.076[a]

Boldface type indicates a statistically significant difference between the groups.
[a]Values were generated with the Mann-Whitney test; all the other values were generated with the Welch test.
[b]Based on protein electrophoresis, paraproteins were not present.
Values are given in the following format: mean; median [min–max]. Missing data due to unsuccessful measurement(s): blood counts – 1 patient with CeD, erythrocyte sedimentation rate – 2 patients with CeD, coagulatory parameters – 1 patient with CeD and 1 control subject; vitamin B_{12} level – 3 patients with CeD and 7 control subjects.
γ-GT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTI, activated partial thromboplastin time; AST, aspartate aminotransferase; CeD, celiac disease; CRP, C-reactive protein; CV, cardiovascular; ESR, erythrocyte sedimentation rate; HDL, high-density lipoprotein; INR, international normalized ratio; LDL, low-density lipoprotein; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red cell distribution width.

RESULTS

Characteristics of the study population

A total of 162 consecutive potential participants were screened for eligibility, 126 of which were included in the study (for flowchart, see Supplementary Digital Content 2, http://links.lww.com/CTG/A419). After matching patients by age and sex (1:1, n = 100), groups were similar in baseline characteristics except for cholesterol profile, eosinophil cell count, and red blood cell distribution width (Tables 2 and 3). For comorbid conditions and medications of the study participants, see Supplementary Digital Content 3, http://links.lww.com/CTG/A420.

Patients with CeD were, on average, aged 31.9 years (range 0–73 years) at diagnosis. Three patients had not started GFD at the time of the study, and all the others were ≥1 year on GFD (median 5.5 years, range: 0.0–36.0 years). At the time of the study, 6 patients (12% of the total) tested positive for urine GIP, 10 patients (20% of the total) scored <8 points on the dietary interview (with median 9 points), and 14 patients (28% of the total) tested positive for tTG or EMA. Patients with CeD rated a

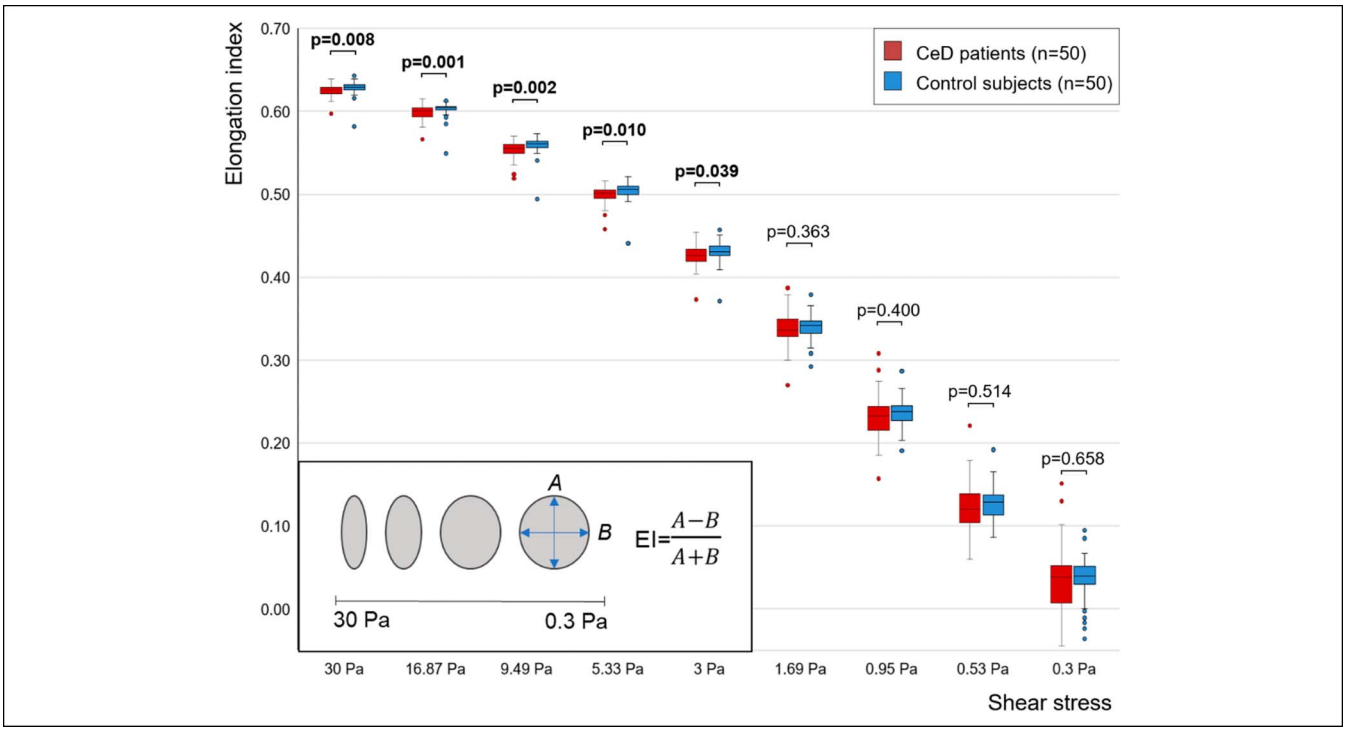

Figure 1. Erythrocyte deformability at different shears (ektacytogram) in patients with celiac disease and control subjects. The horizontal axis indicates the different shears from 0.3 to 30 Pa; the vertical axis shows the elongation index (EI, calculated based on the equation presented in the inlet). Measurements of EI were performed with LORCA. *P* values < 0.05 are highlighted with bold, and were generated with the Mann-Whitney test. Inlet: model for erythrocyte deformation at different shears describing the transition from biconcave to ellipsoid shape. In the equation, A and B represent the long and short axes of the cells, respectively, as indicated with blue arrows in the figure. EI, elongation index (no. of patients = 100).

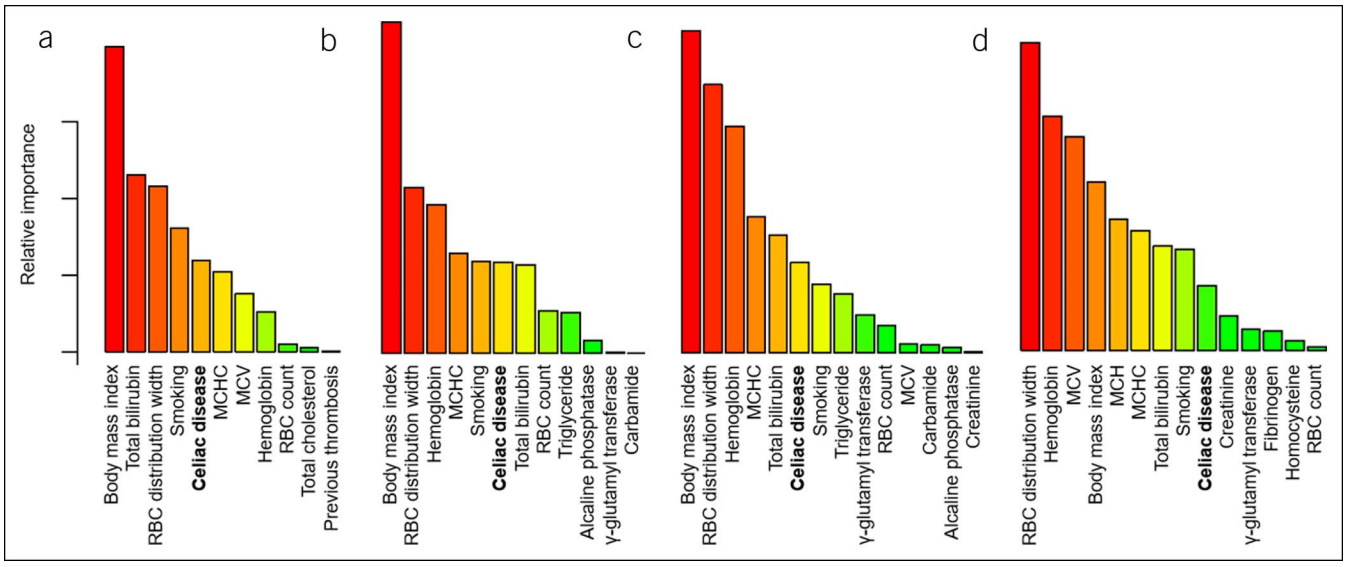

Figure 2. Important predictors of erythrocyte deformability represented by the elongation index at different shears. Panel A: 30 Pa; panel B: 16.87 Pa; panel C: 9.49 Pa; and panel D: 5.33 Pa. Celiac disease is highlighted with bold. The figure was generated with random forest analysis. We imputed 34 covariates, but only those above 0 are displayed because these should be considered important predictors of the outcome. The relative importance is proportional to the height of the bars. MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell (no. of patients = 97).

median of 1.50 points on the Gastrointestinal Symptom Rating Scale.

Control subjects attended a regular checkup (n = 18), were admitted for investigation (n = 16) or mandatory occupational health assessment (n = 16). None of them followed a GFD, and all tested negative for both tTG and EMA.

Hemorheological parameters in patients with celiac disease vs control subjects

Patients with CeD had impaired ED at high shears compared with controls ($P < 0.05$ for the comparisons at 5 shears from 3 to 30 Pa; Figure 1), whereas we observed no significant difference in markers of EA, WBV, PV, and fibrinogen between the groups (for table, see Supplementary Digital Content 4, http://links.lww.com/CTG/A421, which describes the results of comparisons). After correction for 34 clinical and biochemical variables (including CeD vs control status), random forest analysis indicated that CeD is an important determinant of ED at high shears from 5.33 to 30 Pa (Figure 2), unlike at lower shears or regarding EA, HTC, WBV, and PV (for figure, see Supplementary Digital Content 5, http://links.lww.com/CTG/A422, which displays the results of random forest analysis). The most important predictors of hemorheological parameters overlapped those known from the literature, ensuring the validity of random forest analysis.

Association of dietary adherence and serological findings with hemorheological parameters

Supplementary Digital Content 6 (http://links.lww.com/CTG/A423) summarizes raw data and statistics on findings on serological assessment, dietary interview, and urine GIP detection.

ED at high shears seemed to be impaired both in patients with CeD who were seropositive and seronegative (Figure 3a) and both in patients with CeD with poor and good dietary adherence (Figure 3B) compared with control subjects. However, ED at high shears did not differ significantly between seropositive and seronegative patients with CeD (Figure 3a), whereas those with good adherence had better ED only at EI_{30pa} compared with those with poor adherence (which was not supported by the results of urine GIP measurement) (Figure 3b,c). These suggest that the impairment in ED at high shears is independent of the EMA/tTG-mediated immune response and only partly dependent on dietary adherence (favoring a better GFD). Interestingly, ED at low shears did not differ across groups irrespective of findings on serological assessment and dietician-reported dietary adherence (Figure 3d,e). Opposingly, urine GIP+ CeD patients had significantly impaired ED compared with urine GIP− CeD patients and control subjects (Figure 3f) without a difference between urine GIP− CeD patients and control subjects. These results suggest that ED at low shears may not be influenced by EMA/tTG-mediated immune response but may be influenced by other effects of gluten or related proinflammatory reaction.

EA seemed to be significantly impaired in patients with CeD with poor adherence compared with those with good adherence and control subjects (Figure 4). The association applies to AI, $t_{1/2}$, and γ consistently (adjusted P values <0.01 for all; for figure, see Supplementary Digital Content 7, http://links.lww.com/CTG/A424), suggesting prothrombotic alterations in patients with CeD with poor adherence. However, seropositive patients did not differ from seronegative ones so that EMA/tTG-mediated immune response is unlikely explaining the results.

Although WBV seemed to be lower in patients with CeD with good adherence compared with patients with CeD with poor adherence, none of the groups differed significantly from control subjects. HTC, WBV, and PV did not seem to be different substantially across the groups. For figures, see Supplementary Digital Content 7, http://links.lww.com/CTG/A424.

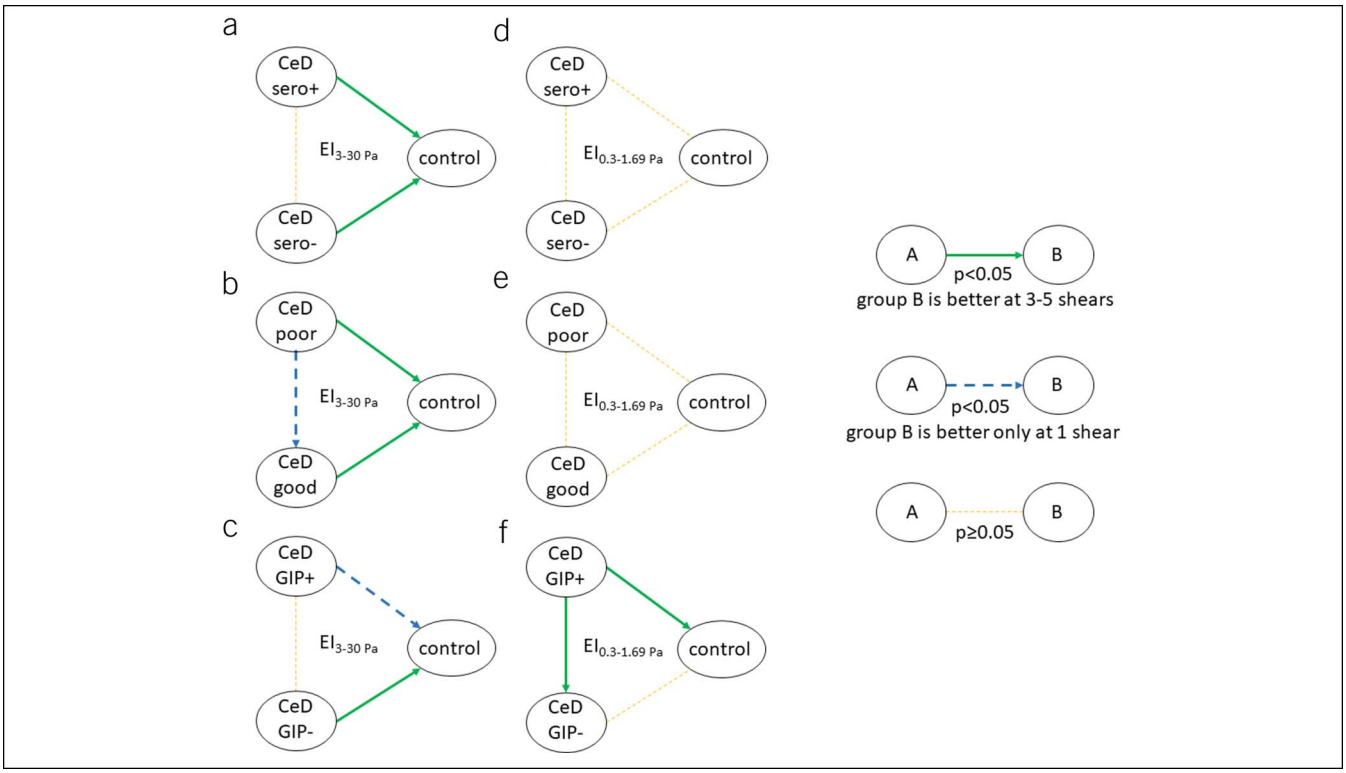

Figure 3. Association of erythrocyte deformability with the CeD-specific serology, adherence based on dietary interview, and urine GIP measurement. Erythrocyte deformability is represented by the elongation index. Panels A and D: serology (based on EMA-IgA/G and tTG-IgA/G) and erythrocyte deformability at low and high shears, respectively. Panels B and E: adherence estimated through dietary interview and erythrocyte deformability at low and high shears, respectively. Panels C and F: adherence estimated through urine GIP detection and erythrocyte deformability at low and high shears, respectively. *P* values were adjusted for multiplicity. Green solid lines represent $P < 0.05$ at 3–5 shears favoring the group at the arrow tip. Blue dashed lines represent $P < 0.05$ at 1 shear favoring the group at the arrow tip. Yellow dashed lines represent no significant difference between groups. CeD, celiac disease; EI, elongation index; EMA, endomysial antibody; GIP, gluten immunogenic peptide; tTG, tissue transglutaminase antibody (no. of patients = 100).

Natural anticoagulants

Measuring the endogenous anticoagulants, we observed no difference in protein C, protein S, and antithrombin activity between the CeD and control groups (Table 4), while CeD proved not to be an important predictor in random forest analysis. Dividing patients by findings on serological assessment, dietician-reported dietary adherence, or by results of urine GIP measurement did not reveal any difference across the groups.

DISCUSSION

We identified impaired ED in CeD, which proved to be independent of findings on serological assessment and dependent, though only partly, on dietary adherence. Also, we found prothrombotic alterations in markers describing EA (AI, $t_{1/2}$, and γ), especially in patients with poor dietary adherence. In addition, we observed lower WBV in patients with CeD with good dietary adherence compared with those with poor adherence. These findings contribute to the understanding of the mechanism of increased CV risk in CeD despite the absence of traditional risk factors: those with CeD have a lower body mass index, less smoking, lower diastolic blood pressure, and a lower level of cholesterol (36,37).

ED at high shears (3–30 Pa) rather models the pressure in the small arteries and arterioles, whereas ED at low shears (0.3–3 Pa) rather models that in the microcirculation. Rigid red blood cells (RBCs) may increase total peripheral resistance and require a higher pressure gradient to squeeze through capillaries. ED is determined mainly by membrane fluidity and cytoplasmatic viscosity, but extracorpuscular factors, such as splenic function or mechanical stress in the narrow capillaries, may have an irreversible impact on RBCs' membrane (14,38). A potential explanation for the impaired ED in CeD might be functional hyposplenism (affecting 16–77% of patients with CeD) when rigid RBCs are no longer removed from the circulation (39,40). Another option may be increased intracellular oxidative stress and reduced nitric oxide production (41). ED might be influenced by comorbidities and lifestyle factors as well (38), however, matching balanced major covariates between the groups. Of note, patients with CeD had lower total and low-density lipoprotein cholesterol levels in our study and previous research (37). Membrane cholesterol content is known to correlate with ED inversely (i.e., the higher the cholesterol, the more rigid the RBCs are) (42). Surprisingly, patients with CeD had impaired ED despite the lower cholesterol level (which is the so-called opposing bias supporting a cause-effect relationship), implying that from a hemorheological aspect, a lower cholesterol level should be considered optimal in CeD. This highlights the need for optimization of calorie density and suboptimal lipid profile of GFD.

Reports showed that an impaired ED is often found with CV diseases (43–45). In our study, at high shears, ED was impaired compared with control irrespective of seropositivity or dietary adherence, whereas patients with good dietician-reported adherence had better ED only at 30 Pa compared with those with poor dietary adherence (based on the dietary interview). This suggests that the impairment is independent on EMA/tTG-mediated

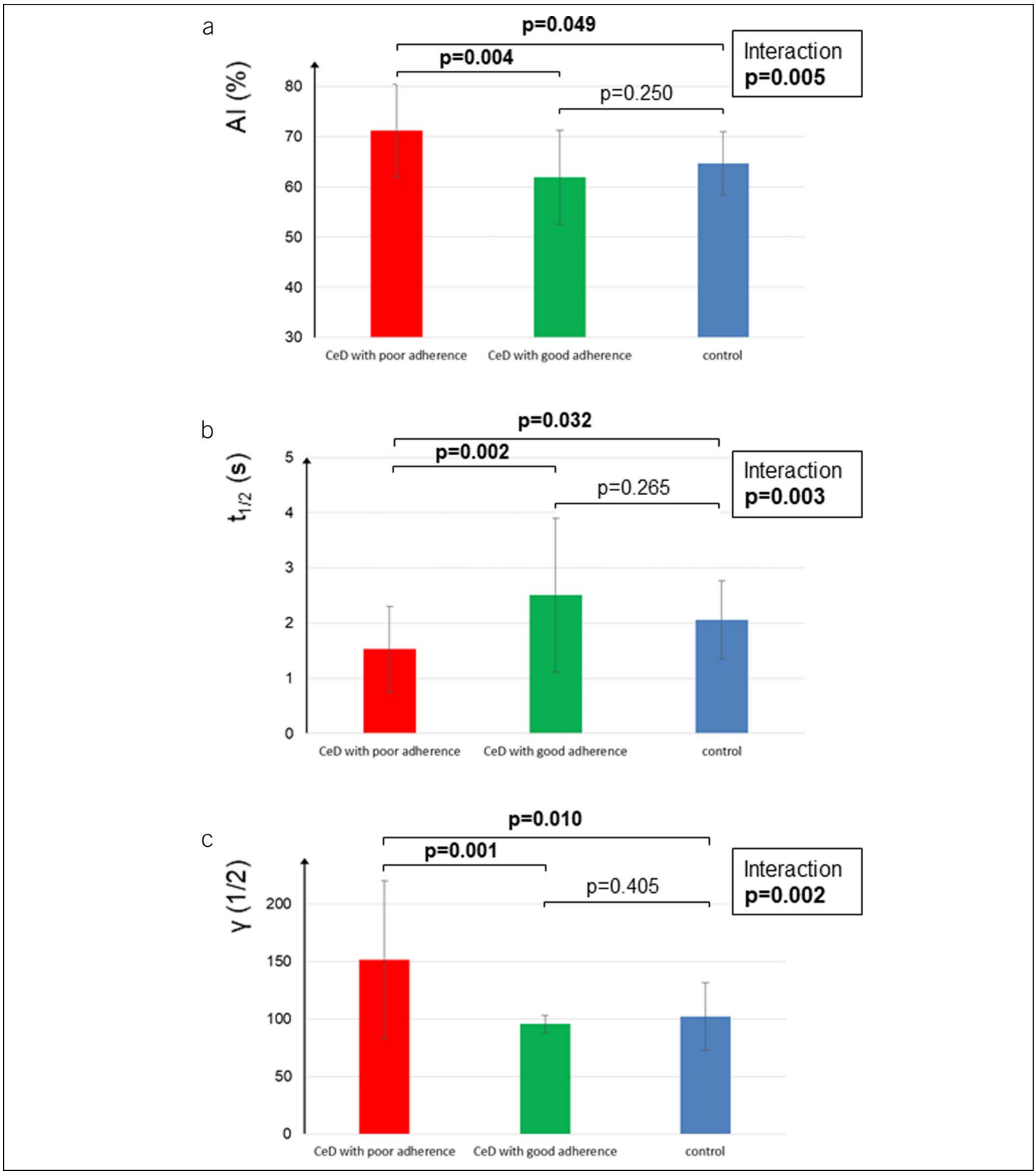

Figure 4. Association of erythrocyte aggregation with dietician-reported dietary adherence. Panel A: aggregation index across groups. Panel B: aggregation half-time across groups. Panel C: threshold shear rate across groups. *P* values were generated after logarithmic transformation of the data and were adjusted for multiplicity in the analysis. AI, aggregation index (no. of patients = 100); CeD, celiac disease.

immune reactions and only loosely dependent on gluten intake. Interestingly, GIP+ CeD patients, compared with GIP− CeD patients, had impaired ED at low shears, whereas we did not observe any difference across the groups if the patients were divided by findings on serological assessment or by the results of dietary interview. Theoretically, gluten, alone or via the activation of an immune response, may cause endothelial damage/activation via oxidative stress or other mechanisms (41,46,47), which can

Table 4. Natural anticoagulants

	Celiac 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 ± SD. *P* values were generated with the Welch test after logarithmic transformation in the case of antithrombin and protein S activity. Missing data due to unsuccessful measurement(s) in the case of 1 patient with CeD and 1 control subject.
CeD, celiac disease.

contribute to the mechanical damage of RBCs when passing through capillaries. In addition, altered endothelium-RBC-platelet interactions can facilitate thrombus formation. The associations about the impaired ED were established in a relatively young population. Considering age- and comorbidity-related changes (e.g., the metabolic syndrome, often seen in patients with CeD on GFD nowadays), further impairment of ED is expected with aging, contributing to the increased CV risk. This warns us of the importance of prevention and treatment of conditions associated with higher CV risk, such as the metabolic syndrome, in CeD. Nevertheless, low-grade (often conflicting) evidence from human clinical trials suggests that physical exercise (48), cholesterol-lowering drugs (42,49), fish oil (50), or polyphenols (51,52) can favorably change ED. Besides, encouragement of patients with CeD to commit to a healthy lifestyle (e.g., quitting smoking and maintaining optimal body weight) should become an organic part of counseling at regular follow-up visits.

In line with ED, EA is essential to maintain normal microcirculation and thrombus formation. EA is mainly determined by plasmatic (fibrinogen and other proteins) and cellular factors (e.g., membrane proteins) (12). Patients with CeD with poor dietary adherence showed prothrombotic alteration, but it seems that patients with CeD can restore normal EA with a strict GFD. Because GIPs, the derivatives of gliadin, are the triggers of the prothrombotic immune response in CeD, dietary transgressions might lead to a release in inflammatory proteins, known to affect EA (53). Because seropositivity does not seem to affect EA, this reaction may be independent of the EMA/tTG-mediated immune response. The prothrombotic changes in EA may be manifested in an increased WBV in patients with CeD with poor dietary adherence compared with those with good dietary adherence (based on the dietary interview). At the same time, HTC and fibrinogen, as important determinants of WBV, were similar across groups. Importantly, WBV was implicated to be associated with CV-related mortality (13).

The role of natural anticoagulants in thrombus formation is beyond dispute (54). Although we observed a lower activity of protein C and protein S compared with control subjects, the difference did not attain statistical significance, nor in subgroup analyses by serological assessment or dietician-reported dietary adherence. Interestingly, lupus anticoagulant was confirmed positive in 6 patients with CeD and 7 control subjects, and only sporadic cases were positive for antibodies against β2-glycoprotein, prothrombin, and cardiolipin, not supporting the theory about thrombophilic autoantibodies in CeD (6).

Strengths of the study include its novelty: to our best knowledge, no comparative study has assessed the hemorheological and natural anticoagulant profile of patients with CeD. In addition, the rigorous, standard methodology and comprehensive analysis should be mentioned. Our multimodal approach of dietary adherence included a dietary interview, celiac-specific serology, and measurement of urine GIP. Nevertheless, we must mention several limitations of the evidence. Prospective cohort studies recording the changes from diagnosis until follow-up visit provide better evidence of a cause-effect relationship. Per the recent guidelines, we do not perform follow-up biopsy routinely, and, consequently, we were unable to test the associations between intestinal histology and test results. However, mucosal changes do not necessary reflect dietary adherence (1,55) and are not necessarily associated with long-term CV outcomes (56).

Our conclusions are rather generalizable to younger and treated patients with CeD since 47 of 50 cases were on GFD ≥1 year (as reflected by the restored homocysteine level). It must be noted, however, that the length of GFD seems not important regarding our outcomes based on the results of random forest analyses.

We found impaired ED in CeD, which seemed to be independent of findings on serological assessment and only partially dependent on dietary adherence. Patients with CeD with poor dietary adherence exhibited prothrombotic alterations of EA, whereas HTC, WBV, PV, and natural anticoagulants seemed not to be substantially affected in CeD irrespective of dietary adherence. These findings should be validated in prospective cohort studies.

The unfavorable alterations of ED and EA can contribute to the elevated CV risk and highlight the importance of CV prevention in CeD during GFD in which good dietary adherence is of utmost importance. As metabolic syndrome has become a threat in CeD and its components (obesity, hypertension, diabetes mellitus, and dyslipidemia) can further aggravate hemorheological status, lifestyle changes (e.g., regular exercise and energy-optimized diet) or even pharmacological interventions (e.g., polyphenols and statins) may help to mitigate hemorheological alterations and thereby reduce CV risk. Randomized studies are called for to validate the clinical implications of our findings.

CONFLICTS OF INTEREST

Guarantor of the article: Judit Bajor, MD, PhD.
Specific author contributions: Z.S., P.H., and J.B. conceptualized the study and drafted the manuscript. J.B. and Á.V. formally screened and consented study participants. Z.S., A.E., B.C., M.N., and K.M. administered the questionnaires and collected and validated the data. Measurements were performed and interpreted by B.C., P.K., and K.T. regarding hemorheological parameters, by A.H. and M.T.F. regarding hemostatic parameters, and by T.B. regarding immunological indicators. N.F. performed the statistical analysis. A.S. and K.M. coordinated the project. Á.V., K.T., P.H., and J.B. supervised the project. All authors revised the draft of the manuscript.
Financial support: This study was supported by an Economic Development and Innovation Operative Programme Grant (GINOP 2.3.2.-15-2016-00048) and a Human Resources Development Operative Programme Grant (EFOP-3.6.2-16-2017-00006) of the National Research, Development and Innovation Office, Hungary, and by the ÚNKP-18-3-I and ÚNKP-19-3-I New National Excellence Program of the Ministry of Human Capacities, Hungary.
Potential competing interests: None to report.
Trial registration number: ISRCTN49677481, ISRCTN Registry.

ACKNOWLEDGEMENTS

We express our gratitude to Dr Barbara Sándor, Kornélia F. Tapasztóné, and Beáta Horváth for the assistance in the measurement and interpretation of hemorheological and hemostatic laboratory parameters and to Ibolya Veczák for conducting the dietary interviews.

Study Highlights

WHAT IS KNOWN

- ✓ Risk of thrombotic events is higher among patients with celiac disease.
- ✓ Hemorheological and hemostatic alterations are potential contributors to cardiovascular events.

WHAT IS NEW HERE

- ✓ Erythrocyte deformation is impaired in patients with celiac disease, which is only partly dependent on dietary adherence.
- ✓ Erythrocyte aggregation is shifted toward a prothrombotic direction in patients with celiac disease with poor dietary adherence.

TRANSLATIONAL IMPACT

- ✓ The prothrombotic hemorheological alterations highlight the importance of cardiovascular prevention in celiac disease.
- ✓ Cardiovascular risk factors (e.g., metabolic syndrome) developing during gluten-free diet may further aggravate hemorheological status.
- ✓ Good dietary adherence can improve hemorheological alterations, but some parameters are independent of diet.

REFERENCES

1. Al-Toma A, Volta U, Auricchio R, et al. European Society for the Study of Coeliac Disease (ESsCD) guideline for coeliac disease and other gluten-related disorders. United Eur Gastroenterol J 2019;7:583–613.
2. Singh P, Arora A, Strand TA, et al. Global prevalence of celiac disease: Systematic review and meta-analysis. Clin Gastroenterol Hepatol 2018; 16:823–36.e2.
3. Tio M, Cox MR, Eslick GD. Meta-analysis: Coeliac disease and the risk of all-cause mortality, any malignancy and lymphoid malignancy. Aliment Pharmacol Ther 2012;35:540–51.
4. Lebwohl B, Green PHR, Söderling J, et al. Association between celiac disease and mortality risk in a Swedish population. JAMA 2020;323: 1277–85.
5. Dumic I, Martin S, Salfiti N, et al. Deep venous thrombosis and bilateral pulmonary embolism revealing silent celiac disease: Case report and review of the literature. Case Rep Gastrointest Med 2017;2017:5236918.
6. Lerner A, Blank M. Hypercoagulability in celiac disease: An update. Autoimmun Rev 2014;13:1138–41.
7. Fry L, Madden AM, Fallaize R. An investigation into the nutritional composition and cost of gluten-free versus regular food products in the UK. J Hum Nutr Diet 2018;31:108–20.
8. Miranda J, Lasa A, Bustamante MA, et al. Nutritional differences between a gluten-free diet and a diet containing equivalent products with gluten. Plant Foods Hum Nutr 2014;69:182–7.
9. Taetzsch A, Das SK, Brown C, et al. Are gluten-free diets more nutritious? An evaluation of self-selected and recommended gluten-free and gluten-containing dietary patterns. Nutrients 2018;10:1881.
10. Vici G, Belli L, Biondi M, et al. Gluten free diet and nutrient deficiencies: A review. Clin Nutr 2016;35:1236–41.
11. Potter MDE, Brienesse SC, Walker MM, et al. Effect of the gluten-free diet on cardiovascular risk factors in patients with coeliac disease: A systematic review. J Gastroenterol Hepatol 2018;33:781–91.
12. Baskurt OK, Meiselman HJ. Erythrocyte aggregation: Basic aspects and clinical importance. Clin Hemorheol Microcirc 2013;53:23–37.
13. Peters SA, Woodward M, Rumley A, et al. Plasma and blood viscosity in the prediction of cardiovascular disease and mortality in the Scottish Heart Health Extended Cohort Study. Eur J Prev Cardiol 2017;24:161–7.
14. Schmid-Schönbein H. Microrheology of erythrocytes, blood viscosity, and the distribution of blood flow in the microcirculation. Int Rev Physiol 1976;9:1–62.
15. Cecchi E, Giglioli C, Valente S, et al. Role of hemodynamic shear stress in cardiovascular disease. Atherosclerosis 2011;214:249–56.
16. Ernst E, Koenig W. Hemorheology, thrombogenesis, and atherosclerosis. Semin Thromb Hemost 1993;19:99–103.
17. Toth A, Papp J, Rabai M, et al. The role of hemorheological factors in cardiovascular medicine. Clin Hemorheol Microcirc 2014;56:197–204.
18. Ernst E, Hein A, Meurer M, et al. Blood rheology in lupus erythematosus. Ann Rheum Dis 1991;50:710–2.
19. Santos MJ, Pedro LM, Canhão H, et al. Hemorheological parameters are related to subclinical atherosclerosis in systemic lupus erythematosus and rheumatoid arthritis patients. Atherosclerosis 2011;219:821–6.
20. Luquita A, Urli L, Svetaz MJ, et al. Erythrocyte aggregation in rheumatoid arthritis: Cell and plasma factor's role. Clin Hemorheol Microcirc 2009; 41:49–56.
21. Akman T, Akarsu M, Akpinar H, et al. Erythrocyte deformability and oxidative stress in inflammatory bowel disease. Dig Dis Sci 2012;57: 458–64.
22. Lobo AJ, Jones SC, Juby LD, et al. Plasma viscosity in inflammatory bowel disease. J Clin Pathol 1992;45:54–7.
23. Maharshak N, Arbel Y, Shapira I, et al. Increased strength of erythrocyte aggregates in blood of patients with inflammatory bowel disease. Inflamm Bowel Dis 2009;15:707–13.
24. Moran A, Jones A, Asquith P. Laboratory markers of colonoscopic activity in ulcerative colitis and Crohn's colitis. Scand J Gastroenterol 1995;30:356–60.
25. Novacek G, Vogelsang H, Genser D, et al. Changes in blood rheology caused by Crohn's disease. Eur J Gastroenterol Hepatol 1996;8:1089–93.
26. Yakar T, Cosar AM, Gokturk HS, et al. Plasma viscosity: A potential predictor of both medical treatment response and clinical stage of ulcerative colitis. Ann Ital Chir 2016;87:531–43.
27. Zilberman L, Rogowski O, Rozenblat M, et al. Inflammation-related erythrocyte aggregation in patients with inflammatory bowel disease. Dig Dis Sci 2005;50:677–83.
28. 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:e026315.
29. Vandenbroucke JP, von Elm E, Altman DG, et al. Strengthening the reporting of observational studies in epidemiology (STROBE): Explanation and elaboration. PLoS Med 2007;4:e297.
30. Rubio-Tapia A, Hill ID, Kelly CP, et al. ACG clinical guidelines: Diagnosis and management of celiac disease. Am J Gastroenterol 2013;108:656–77.
31. Husby S, Koletzko S, Korponay-Szabó IR, et al. European society for pediatric gastroenterology, hepatology, and nutrition guidelines for the diagnosis of coeliac disease. J Pediatr Gastroenterol Nutr 2012;54:136–60.
32. Svedlund J, Sjödin I, Dotevall G. GSRS: A clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci 1988;33:129–34.
33. Baskurt OK, Boynard M, Cokelet GC, et al. New guidelines for hemorheological laboratory techniques. Clin Hemorheol Microcirc 2009; 42:75–97.
34. de Lourdes Moreno M, Rodríguez-Herrera A, Sousa C, et al. Biomarkers to monitor gluten-free diet compliance in celiac patients. Nutrients 2017;9:46.
35. Strobl C, Boulesteix AL, Kneib T, et al. Conditional variable importance for random forests. BMC Bioinformatics 2008;9:307.
36. Brar P, Kwon GY, Holleran S, et al. Change in lipid profile in celiac disease: Beneficial effect of gluten-free diet. Am J Med 2006;119:786–90.
37. West J, Logan RF, Hill PG, et al. Seroprevalence, correlates, and characteristics of undetected coeliac disease in England. Gut 2003;52:960–5.
38. Radosinska J, Vrbjar N. The role of red blood cell deformability and Na,K-ATPase function in selected risk factors of cardiovascular diseases in humans: Focus on hypertension, diabetes mellitus and hypercholesterolemia. Physiol Res 2016;65(Suppl 1):S43–S54.
39. Di Sabatino A, Rosado MM, Cazzola P, et al. Splenic hypofunction and the spectrum of autoimmune and malignant complications in celiac disease. Clin Gastroenterol Hepatol 2006;4:179–86.
40. William BM, Corazza GR. Hyposplenism: A comprehensive review. Part I: Basic concepts and causes. Hematology 2007;12:1–13.

41. Moretti S, Mrakic-Sposta S, Roncoroni L, et al. Oxidative stress as a biomarker for monitoring treated celiac disease. Clin Transl Gastroenterol 2018;9:157.
42. Kohno M, Murakawa K, Yasunari K, et al. Improvement of erythrocyte deformability by cholesterol-lowering therapy with pravastatin in hypercholesterolemic patients. Metabolism 1997;46:287–91.
43. Fornal M, Korbut RA, Królczyk J, et al. Left ventricular geometry and rheological properties of erythrocytes in patients at cardiovascular disease risk. Clin Hemorheol Microcirc 2009;43:203–8.
44. Penco M, Romano S, Dagianti A Jr, et al. Modifications of whole blood filterability during acute myocardial infarction. Clin Hemorheol Microcirc 2000;22:153–9.
45. Yoshida H, Satoh K, Takamatsu S. Platelet-activating factor acetylhydrolase in red cell membranes. Does decreased activity impair erythrocyte deformability in ischemic stroke patients? Stroke 1993;24:14–8.
46. Diaz-Castro J, Muriel-Neyra C, Martin-Masot R, et al. Oxidative stress, DNA stability and evoked inflammatory signaling in young celiac patients consuming a gluten-free diet. Eur J Nutr 2019;59:1577–1584.
47. Dolfini E, Elli L, Roncoroni L, et al. Damaging effects of gliadin on three-dimensional cell culture model. World J Gastroenterol 2005;11:5973–7.
48. Mika P, Spodaryk K, Cencora A, et al. Red blood cell deformability in patients with claudication after pain-free treadmill training. Clin J Sport Med 2006;16:335–40.
49. Szapary L, Horvath B, Marton Z, et al. Short-term effect of low-dose atorvastatin on haemorrheological parameters, platelet aggregation and endothelial function in patients with cerebrovascular disease and hyperlipidaemia. CNS Drugs 2004;18:165–72.
50. Cartwright IJ, Pockley AG, Galloway JH, et al. The effects of dietary omega-3 polyunsaturated fatty acids on erythrocyte membrane phospholipids, erythrocyte deformability and blood viscosity in healthy volunteers. Atherosclerosis 1985;55:267–81.
51. Magyar K, Halmosi R, Palfi A, et al. Cardioprotection by resveratrol: A human clinical trial in patients with stable coronary artery disease. Clin Hemorheol Microcirc 2012;50:179–87.
52. Radosinska J, Horvathova M, Frimmel K, et al. Acute dark chocolate ingestion is beneficial for hemodynamics via enhancement of erythrocyte deformability in healthy humans. Nutr Res 2017;39:69–75.
53. Weng X, Roederer GO, Beaulieu R, et al. Contribution of acute-phase proteins and cardiovascular risk factors to erythrocyte aggregation in normolipidemic and hyperlipidemic individuals. Thromb Haemost 1998; 80:903–8.
54. Soare AM, Popa C. Deficiencies of proteins C, S and antithrombin and activated protein C resistance–their involvement in the occurrence of Arterial thromboses. J Med Life 2010;3:412–5.
55. Szakács Z, Mátrai P, Hegyi P, et al. Younger age at diagnosis predisposes to mucosal recovery in celiac disease on a gluten-free diet: A meta-analysis. PLoS One 2017;12:e0187526.
56. Szakács Z, Gede N, Gyöngyi Z, et al. A call for research on the prognostic role of follow-up histology in celiac disease: A systematic review. Front Physiol 2019;10:1408.

BMJ Open

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

Zsolt Szakács,[1,2,3] Beáta Csiszár,[1,4] Péter Kenyeres,[1,4] Patrícia Sarlós,[1,5] Bálint Erőss,[3,5] Alizadeh Hussain,[1,6] Ágnes Nagy,[6] Balázs Kőszegi,[7] Ibolya Veczák,[5] Nelli Farkas,[8] Emőke Bódis,[3] Katalin Márta,[1,3] Andrea Szentesi,[3] Margit Tőkés-Füzesi,[9] Tímea Berki,[10] Áron Vincze,[1,2,5] Kálmán Tóth,[1,4] Péter Hegyi,[1,2,3] Judit Bajor[2,5]

To cite: 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**:e026315. doi:10.1136/bmjopen-2018-026315

► Prepublication history and additional material for this paper are available online. To view these files, please visit the journal online (http://dx.doi.org/10.1136/bmjopen-2018-026315).

PH and JB contributed equally.

Received 29 August 2018
Revised 18 December 2018
Accepted 11 February 2019

For numbered affiliations see end of article.

Correspondence to
Dr Péter Hegyi;
p.hegyi@tm-centre.org

ABSTRACT

Introduction Haemorheological and haemostatic changes predispose to the development of arterial and venous thrombotic events; however, limited information is available on the status of these changes in coeliac disease (CeD) and inflammatory bowel disease (IBD). In this study, we aim to describe the haemorheological and haemostatic 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-matched 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, haemorheological (ie, erythrocyte deformability and aggregation, viscosity of whole blood and plasma) and haemostatic parameters (eg, protein C, protein S and antithrombin) with immunological indicators (ie, coeliac-specific serology and antiphospholipid antibodies) will be measured from venous blood for every participant. Primary and secondary outcomes will be haemorheological and haemostatic 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 number ISRCTN49677481.

Strengths and limitations of this study

- ► Immune-mediated bowel diseases are associated with an increased risk of arterial and venous thrombosis, but specific haemorheological and haemostatic alterations are understudied in coeliac disease (CeD) and incomplete in inflammatory bowel disease (IBD).
- ► This case–control study prospectively recruits newly diagnosed and followed-up cases of CeD and IBD with age-matched 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 haemorheological (ie, erythrocyte aggregation and deformability, plasma and whole blood viscosity), haemostatic parameters (eg, levels of fibrinogen, prothrombin time, protein C, protein S and antithrombin) and immunological indicators (eg, coeliac-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. Coeliac 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 on 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 haemorheological[9–11] and haemostatic 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 haemorheological profile as well as the altered levels or function of pro-coagulant 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 haemostatic alterations in a small cohort of patients: sporadic cases of protein C and protein S deficiency (due to vitamin K malabsorption), hyperhomocysteinaemia, and antiphospholipid antibodies were identified.[26] No studies have assessed the haemorheological changes in CeD. The multifactorial aetiology of thrombosis may embrace the interplay of malabsorption (vitamin and mineral deficiencies, eg, vitamin B_{12} and K deficiency), thrombophilic autoantibodies [anti-tissue transglutaminase (tTG) and antiphospholipid antibodies], hyperhomocysteinaemia, 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, ie, stroke and myocardial infarction.[33 34] A significant decline in anticoagulant mechanism is well-established and there are sporadic reports on activity-dependent prothrombotic haemorheological changes.[35–38] However, while individual studies have focused on single outcomes of laboratory parameters, none of them have assessed the complete haemorheological profile of patients.[39–42] Other risk factors include immobilisation, surgical interventions, glucocorticoid therapy, vitamin deficiencies, hyperhomocysteinaemia 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 haemorheological and haemostatic parameters within a study to provide an overall view of thrombotic risk. Since our knowledge of haemorheological and haemostatic 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 haemorheological and haemostatic alterations and two common immune-mediated diseases (CeD and IBD).
2. Secondary objective.
   - To investigate the effect of disease activity on the haemorheological and haemostatic profiles of CeD and IBD patients.
   - To find an association between the dietary adherence of CeD patients and the haemorheological and haemostatic alterations.
   - To assess the modifying effect of immunosuppressant drugs in IBD on the haemorheological and haemostatic 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 WHO checklist, see table 1). The study protocol was planned in accordance with the Standard Protocol Items: Recommendations for Interventional Trials 2013 Statement.[45]

Trial organisation and steering committee

The Centre for Translational Medicine at the University of Pécs, which was established to advance medical research in gastroenterology, is the co-ordinator 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 organising patient recruitment,

Table 1 WHO 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-3.6.3-VEKOP-16-2017-00009; Translational Medicine Foundation; and New National Excellence Programme, Ministry of Human Capacities (ÚNKP-17–3-II, ÚNKP-18–3-I)
Primary sponsor	None
Secondary sponsor(s)	None
Contact for public queries	Zsolt Szakács, MD, szakacs.zsolt@pte.hu
Contact for scientific queries	Judit Bajor, MD, bajor.judit@pte.hu
Public title	Investigation of haemorheological and haemostatic alterations in coeliac disease and inflammatory bowel disease in comparison with healthy subjects: A case–control study (HERMES)
Scientific title	Haemorheological and haemostatic alterations in coeliac disease and inflammatory bowel disease in comparison with non-coeliac, non-IBD subjects: A case–control study (HERMES)
Countries of recruitment	Hungary
Health condition(s) or problem(s) studied	Coeliac disease and inflammatory bowel disease
Intervention(s)	Questionnaires (thrombophilia, dietary adherence, disease activity), urine collection (dietary adherence—urine-gluten immunogenic peptide detection), blood collection (haemorheological, haemostatic 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 coeliac disease (by ESPHGAN, ACG, WGO guidelines), or from inflammatory bowel disease (by ECCO guidelines), and non-coeliac, 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 coeliac 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)	Haemorheological test results
Key secondary outcomes	Haemostatic test results

IBD, inflammatory bowel disease.

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 (European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPHGAN) and American College of Gastroenterology (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 (European Crohn's and Colitis Organisation (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 the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula is <60 mL/min/1.73 m^2 (CKD3 or more severe kidney failure).
    - Liver cirrhosis in Child–Pugh B–C.
    - Heart failure (New York Heart Association (NYHA) III–IV).
    - Active malignant diseases.
  - Any acute diseases or invasive procedures within 2 weeks of recruitment (eg, 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 centre 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 1 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.

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

Table 2 Schedule for the study

	Study period				
	Enrolment	Allocation	Post-allocation		
Time point	−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*					≥†

*After analysis, blood and urine residues will be stored in the biobank.
†Samples will be deep frozen until all participants have been recruited.

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	+	+	+
Haemorheology	+	+	+
Haemostasis	+	+	+
Immunological indicators	+	+	+

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

to their general practitioners or a specialist for further investigation and management.

Measurements

All samples will be collected and questionnaires will be administered within 2 hours after allocation. Actions for each group are defined and listed in table 3.

Detailed history (including medications for preceding 3 months) and risk factors of venous and arterial thrombotic events will be covered with a 15 min 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 1–7 (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 1–10, (2) self-reporting,[54] (3) a test measuring knowledge of gluten-free foods, (4) urine gluten-immunogenic peptides (GIP) detection (details in the text) and (5) coeliac-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 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/IgA/IgM, B2-glycoprotein-I IgG/IgA/IgM, prothrombin IgG/IgA/IgM) and coeliac-specific antibodies (tTG IgA/IgG, EMA IgA).
- Haemostatic parameters: prothrombin, thrombin time, activated partial thromboplastin time, fibrinogen, antithrombin activity, protein C activity and protein S activity.
- Haemorheological 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 online supplementary material.

Strict adherence will be kept during the haemorheological tests to the guidelines proposed by the International Expert Panel for Standardisation of Haemorheological Methods.[56] The fact that equipment for haemorheological measurements is not available in other centres in Hungary and that blood samples must be processed within 2 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 haemostatic measurements (eg, 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.
   - Haemorheological test results (erythrocyte aggregation and deformability, whole blood and plasma viscosity).

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

*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.
N/A, not applicable.

2. Secondary.
   - Haemostatic 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- matched 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.

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 familiarise them with the study requirements, standardised 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 analysed with Student's tests, methods of Variance Analysis, and regression models if data are normally distributed; otherwise, non-parametric tests will be introduced. χ^2 or Fisher's tests will be used to analyse categorical variables. Multivariate analysis will be used to take the most important thrombotic factors into account (eg, the use of oral contraceptives and immunosuppressants, previous thrombotic history, smoking, comorbidities). A probability of less than 0.05 indicates a statistically significant difference between groups.

Only patients with a full dataset in their haemorheological and haemostatic profile will be included in the analysis. The following comparisons will be done: CeD versus control, tTG+CeD versus tTG- CeD, CeD with good dietary adherence versus CeD with poor dietary adherence, IBD versus control, active IBD versus remitting IBD and Crohn's disease versus 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 5 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 CeD and the effect of a gluten-free diet—Coeliac 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 (eg, consequences of malabsorption and chronic inflammation) but it is uncertain whether the thrombotic risk completely normalises.[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 hospitalisation, pregnancy, or immobilisation should be further investigated.

IBD guidelines recommend that thromboprophylaxis should be considered in all inpatients 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 personalised 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

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.

Author affiliations
[1]János Szentágothai Research Center, University of Pécs, Pécs, Hungary
[2]Clinical Medicine Doctoral School, University of Szeged, Szeged, Hungary
[3]Institute for Translational Medicine, Medical School, University of Pécs, Pécs, Hungary
[4]Division of Cardiology and Angiology, First Department of Medicine, University of Pécs, Pécs, Hungary
[5]Division of Gastroenterology, First Department of Medicine, Medical School, University of Pécs, Pécs, Hungary
[6]Division of Hematology, First Department of Medicine, Medical School, University of Pécs, Pécs, Hungary
[7]Department of Biochemistry and Medical Chemistry, Medical School, University of Pécs, Pécs, Hungary
[8]Institute of Bioanalysis, Medical School, University of Pécs, Pécs, Hungary
[9]Department of Laboratory Medicine, Medical School, University of Pécs, Pécs, Hungary
[10]Department of Immunology and Biotechnology, Medical School, University of Pécs, Pécs, Hungary

Contributors JB is the Principal Investigator. ZS is the Trial Co-ordinator. ZS, PH, JB, ÁV and KT conceptualised 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 coeliac disease and inflammatory bowel patients. BC and PK are performing the haemorheological measurements and interpreting the results. AH, ÁN, TB and MT-F 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 coeliac patients. KM, AS, ZS and PH are responsible for data management, administrative co-ordination and biological sampling; they drafted and revised the manuscript. All the authors have read and approved the final manuscript.

Funding The project is non-industry-funded. Study and centre costs are covered by the University of Pécs Medical School, by a Momentum Grant from the Hungarian Academy of Sciences (LP2014-10/2014), by a Highly Cited Publication Grant (KH 125678) from the National Research Development and Innovation Office, by GINOP 2.3.2-15-2016-00048 Stay Alive, EFOP-3.6.2-16-2017-0006, and EFOP-3.6.3-VEKOP-16-2017-00009; and by the Translational Medicine Foundation. In addition, this project is supported by the ÚNKP-17-3-II and ÚNKP-18-3-I New National Excellence Programme, Ministry of Human Capacities. Funders have no influence on preparations, course, interpretation, or publication of results.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was approved by the Regional and Local Research Ethics Committee, University of Pécs (Ref No 6917).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. El-Gabalawy H, Guenther LC, Bernstein CN. Epidemiology of immune-mediated inflammatory diseases: incidence, prevalence, natural history, and comorbidities. *J Rheumatol Suppl* 2010;85:2–10.
2. Pascual V, Dieli-Crimi R, López-Palacios N, *et al*. Inflammatory bowel disease and celiac disease: overlaps and differences. *World J Gastroenterol* 2014;20:4846–56.
3. Bai JC, Ciacci C. World gastroenterology organisation global guidelines: Celiac disease February 2017. *J Clin Gastroenterol* 2017;51:755–68.
4. Ludvigsson JF, Leffler DA, Bai JC, *et al*. The Oslo definitions for coeliac disease and related terms. *Gut* 2013;62:43–52.
5. Kurppa K, Paavola A, Collin P, *et al*. Benefits of a gluten-free diet for asymptomatic patients with serologic markers of celiac disease. *Gastroenterology* 2014;147:610–7.
6. Zhang YZ, Li YY, Yy L. Inflammatory bowel disease: pathogenesis. *World J Gastroenterol* 2014;20:91–9.
7. Molodecky NA, Soon IS, Rabi DM, *et al*. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012;142:46–54.
8. Kaplan GG. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol* 2015;12:720–7.
9. Ernst E, Hein A, Meurer M, *et al*. Blood rheology in lupus erythematosus. *Ann Rheum Dis* 1991;50:710–2.
10. Shurkhina ES, Nesterenko VM, Lisovskaya IL, *et al*. Detection of stages of autoimmune hemolytic anemia by evaluating erythrocyte deformability and density. *Bull Exp Biol Med* 2004;138:280–3.
11. Spengler MI, Svetaz MJ, Leroux MB, *et al*. Erythrocyte aggregation in patients with systemic lupus erythematosus. *Clin Hemorheol Microcirc* 2011;47:279–85.

12. Adams MJ, Palatinus AA, Harvey AM, *et al*. Impaired control of the tissue factor pathway of blood coagulation in systemic lupus erythematosus. *Lupus* 2011;20:1474–83.
13. Costallat LT, Ribeiro CC, Annichino-Bizzacchi JM. Antithrombin, protein S and protein C and antiphospholipid antibodies in systemic lupus erythematosus. *Sangre* 1998;43:345–8.
14. Kordich LC, Forastiero RR, Basilotta E, *et al*. Natural inhibitors of blood coagulation and fibrinolysis in patients with lupus anticoagulant. *Blood Coagul Fibrinolysis* 1992;3:765–72.
15. Zöller B, Li X, Sundquist J, *et al*. Risk of pulmonary embolism in patients with autoimmune disorders: a nationwide follow-up study from Sweden. *Lancet* 2012;379:244–9.
16. Ungprasert P, Wijarnpreecha K, Tanratana P. Risk of venous thromboembolism in patients with celiac disease: A systematic review and meta-analysis. *J Gastroenterol Hepatol* 2016;31:1240–5.
17. Sarlos P, Szemes K, Hegyi P, *et al*. Steroid but not biological therapy elevates the risk of venous thromboembolic events in inflammatory bowel disease: a meta-analysis. *J Crohns Colitis* 2018;12:489–98.
18. Chang HH, Chiang BL. The diagnosis and classification of autoimmune coagulopathy: an updated review. *Autoimmun Rev* 2014;13:587–90.
19. Park KH, Kim U, Choi KU, *et al*. Hemorheologic alterations in patients with type 2 diabetes mellitus presented with an acute myocardial infarction. *Diabetes Metab J* 2018;42:155–63.
20. Vayá A, Rivera L, de la Espriella R, *et al*. Red blood cell distribution width and erythrocyte deformability in patients with acute myocardial infarction. *Clin Hemorheol Microcirc* 2015;59:107–14.
21. Zorio E, Murado J, Arizo D, *et al*. Haemorheological parameters in young patients with acute myocardial infarction. *Clin Hemorheol Microcirc* 2008;39:33–41.
22. Banerjee R, Nageswari K, Puniyani RR. Association of hemorheological parameters and risk of stroke in hypertensives of Indian origin. *Clin Exp Hypertens* 2000;22:687–94.
23. Wolberg AS, Aleman MM, Leiderman K, *et al*. Procoagulant activity in hemostasis and thrombosis: Virchow's triad revisited. *Anesth Analg* 2012;114:275–85.
24. Lerner A, Blank M. Hypercoagulability in celiac disease-an update. *Autoimmun Rev* 2014;13:1138–41.
25. Dumic I, Martin S, Salfiti N, *et al*. Deep venous thrombosis and bilateral pulmonary embolism revealing silent celiac disease: Case report and review of the literature. *Case Rep Gastrointest Med* 2017;2017:1–8.
26. Berthoux E, Fabien N, Chayvialle JA, *et al*. [Adult celiac disease with thrombosis: a case series of seven patients. Role of thrombophilic factors]. *Rev Med Interne* 2011;32:600–4.
27. Lerner A, Agmon-Levin N, Shapira Y, *et al*. The thrombophilic network of autoantibodies in celiac disease. *BMC Med* 2013;11:89.
28. Hallert C, Grant C, Grehn S, *et al*. Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Aliment Pharmacol Ther* 2002;16:1333–9.
29. Shamir R, Shoenfeld Y, Blank M, *et al*. The prevalence of coeliac disease antibodies in patients with the antiphospholipid syndrome. *Lupus* 2003;12:394–9.
30. Johannesdottir SA, Erichsen R, Horváth-Puhó E, *et al*. Coeliac disease and risk of venous thromboembolism: a nationwide population-based case-control study. *Br J Haematol* 2012;157:499–501.
31. Ciaccio EJ, Lewis SK, Biviano AB, *et al*. Cardiovascular involvement in celiac disease. *World J Cardiol* 2017;9:652–66.
32. Verma AK, Gatti S, Galeazzi T, *et al*. Gluten contamination in naturally or labeled gluten-free products marketed in Italy. *Nutrients* 2017;9:115.
33. Mitu O, Alexandrescu DM, Cătălina M, *et al*. Is there a cardiovascular risk in inflammatory bowel diseases? *Rev Med Chir Soc Med Nat Iasi* 2014;118:918–23.
34. Kristensen SL, Ahlehoff O, Lindhardsen J, *et al*. Disease activity in inflammatory bowel disease is associated with increased risk of myocardial infarction, stroke and cardiovascular death-a Danish nationwide cohort study. *PLoS One* 2013;8:e56944.
35. Cakal B, Gokmen A, Yalinkilic M, *et al*. Natural anticoagulant protein levels in Turkish patients with inflammatory bowel disease. *Blood Coagul Fibrinolysis* 2010;21:118–21.
36. Heneghan MA, Cleary B, Murray M, *et al*. Activated protein C resistance, thrombophilia, and inflammatory bowel disease. *Dig Dis Sci* 1998;43:2164–7.
37. Kohoutova D, Pecka M, Cihak M, *et al*. Prevalence of hypercoagulable disorders in inflammatory bowel disease. *Scand J Gastroenterol* 2014;49:287–94.
38. Yurekli BP, Aksoy DY, Aybar M, *et al*. The search for a common thrombophilic state during the active state of inflammatory bowel disease. *J Clin Gastroenterol* 2006;40:809–13.
39. Akman T, Akarsu M, Akpinar H, *et al*. Erythrocyte deformability and oxidative stress in inflammatory bowel disease. *Dig Dis Sci* 2012;57:458–64.
40. Novacek G, Vogelsang H, Genser D, *et al*. Changes in blood rheology caused by Crohn's disease. *Eur J Gastroenterol Hepatol* 1996;8:1089–93.
41. Zilberman L, Rogowski O, Rozenblat M, *et al*. Inflammation-related erythrocyte aggregation in patients with inflammatory bowel disease. *Dig Dis Sci* 2005;50:677–83.
42. Lobo AJ, Jones SC, Juby LD, *et al*. Plasma viscosity in inflammatory bowel disease. *J Clin Pathol* 1992;45:54–7.
43. Danese S, Papa A, Saibeni S, *et al*. Inflammation and coagulation in inflammatory bowel disease: The clot thickens. *Am J Gastroenterol* 2007;102:174–86.
44. Magro F, Soares JB, Fernandes D. Venous thrombosis and prothrombotic factors in inflammatory bowel disease. *World J Gastroenterol* 2014;20:4857–72.
45. Chan AW, Tetzlaff JM, Altman DG, *et al*. SPIRIT 2013 statement: defining standard protocol items for clinical trials. *Ann Intern Med* 2013;158:200–7.
46. Márta K, Szabó AN, Pécsi D, *et al*. High versus low energy administration in the early phase of acute pancreatitis (GOULASH trial): protocol of a multicentre randomised double-blind clinical trial. *BMJ Open* 2017;7:e015874.
47. Husby S, Koletzko S, Korponay-Szabó IR, *et al*. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr* 2012;54:136–60.
48. Rubio-Tapia A, Hill ID, Kelly CP, *et al*. ACG clinical guidelines: diagnosis and management of celiac disease. *Am J Gastroenterol* 2013;108:656–76.
49. Dignass A, Eliakim R, Magro F, *et al*. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *J Crohns Colitis* 2012;6:965–90.
50. Dignass A, Van Assche G, Lindsay JO, *et al*. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. *J Crohns Colitis* 2010;4:28–62.
51. Svedlund J, Sjödin I, Dotevall G. GSRS--a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. *Dig Dis Sci* 1988;33:129–34.
52. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987;317:1625–9.
53. Modigliani R, Mary JY, Simon JF, *et al*. Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. Groupe d'Etude Thérapeutique des Affections Inflammatoires Digestives. *Gastroenterology* 1990;98:811–8.
54. Silvester JA, Weiten D, Graff LA, *et al*. Living gluten-free: adherence, knowledge, lifestyle adaptations and feelings towards a gluten-free diet. *J Hum Nutr Diet* 2016;29:374–82.
55. Hindryckx P, Levesque BG, Holvoet T, *et al*. Disease activity indices in coeliac disease: systematic review and recommendations for clinical trials. *Gut* 2018;67:61–9.
56. Baskurt OK, Boynard M, Cokelet GC, *et al*. New guidelines for hemorheological laboratory techniques. *Clin Hemorheol Microcirc* 2009;42:75–97.
57. Beyrouti R, Mansour M, Kacem A, *et al*. Recurrent cerebral venous thrombosis revealing celiac disease: an exceptional case report. *Acta Neurol Belg* 2017;117:341–3.
58. Szakács Z, Mátrai P, Hegyi P, *et al*. Younger age at diagnosis predisposes to mucosal recovery in celiac disease on a gluten-free diet: A meta-analysis. *PLoS One* 2017;12:e0187526.
59. Gionchetti P, Dignass A, Danese S, *et al*. 3rd European evidence-based consensus on the diagnosis and management of crohn's disease 2016: Part 2: Surgical management and special situations. *J Crohns Colitis* 2017;11:135–49.
60. Nguyen GC, Bernstein CN, Bitton A, *et al*. Consensus statements on the risk, prevention, and treatment of venous thromboembolism in inflammatory bowel disease: Canadian Association of Gastroenterology. *Gastroenterology* 2014;146:835–48.