UNIVERSITY OF LATVIA

FACULTY OF MEDICINE

POĻINA ZAĻIZKO

DETERMINING THE ROLE OF THIOPURINE METABOLISM USING IMMUNOLOGICAL, MOLECULAR BIOLOGY METHODS, AND METABOLIC STATUS IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

DOCTORAL THESIS

Promotion to the degree of Doctor of Clinical medicine (PhD in Medicine)
Subfield of Internal medicine

Riga 2022

This doctoral thesis study was carried out at the Department of Internal Medicine, Faculty of Medicine, University of Latvia, from 2017 to 2022.

The thesis contains an introduction, 4 chapters, a reference list, and a list of the authors' scientific publications.

Form of the thesis: dissertation in medicine

Supervisor: Dr med., Professor **Aldis Puķītis**

Reviewers:

1) Professor **Valdis Pīrāgs**, University of Latvia;

2) Professor **Santa Purviņa**, Riga Stradiņš University;

3) Professor **Limas Kupčinskas**, Lithuanian University of Health Sciences.

The thesis will be defended at the public session of the Doctoral Committee of Medicine and Health Sciences, University of Latvia, on the 29th of April, 2022 at the University of Latvia.

The thesis is available at the Library of the University of Latvia, 19 Raina blvd.

Chairman of the Doctoral Committee ________________/

Dr med., Professor **Valdis Pīrāgs**

Secretary of the Doctoral Committee ________________/

Dr biol., Associated Professor **Līga Plakane**

© University of Latvia, 2022

© Poļina Zaļizko, 2022

ANNOTATION

The number of patients with inflammatory bowel disease (IBD) is increasing worldwide, and Latvia is no exception. IBD has become a prevalent disease in industrialised countries with an increasing incidence over the past 50 years. Thiopurine monitoring is a promising strategy for optimising IBD therapeutics. Despite the increasing adoption of thiopurine metabolism analysis in clinical practice, evidence and overall approach to thiopurine monitoring in IBD management are not available. Azathioprine is used widely for IBD treatment. However, adverse effects have been reported in approximately 10% of IBD patients receiving thiopurines. Enzyme thiopurine methyltransferase (TPMT) plays a significant role in the metabolism of thiopurines, and low TPMT activity is associated with altered thiopurine metabolism, overproduction of cytotoxic metabolites, and myelosuppression. In patients with IBD, TPMT genotyping can be performed prior to treatment to evaluate treatment risks; however, this does not exclude that patients may or may not tolerate thiopurines.

The aim of this Doctoral Thesis was to investigate the interindividual variability of thiopurine metabolism using immunological, molecular biology methods, and metabolic status in patients with inflammatory bowel disease.

We analysed the TPMT genotype and phenotype in IBD patients in Latvia and identified malnutrition parameters associated with more pronounced metabolic status changes in IBD patients as an anticipatory indicator of disease activity.

Our results showed that the frequencies of common TPMT alleles in the Latvia IBD population were similar to those of other European populations. The homozygous wild-type TPMT *1/*1 genotype was the most frequent genotype in UC and CD patients and TPMT *3A was the most prevalent polymorphism.

We recommend that TPMT genotyping or phenotyping should be prioritised for higher-risk patients to help predict thiopurine-induced adverse drug reactions and to determine personalised therapeutic options. IBD patients with a high disease activity index were at a noticeably high risk of malnutrition. Identification of reduction in muscle mass in CD patients can be used as an anticipatory indicator of disease activity.

Keywords: inflammatory bowel disease, thiopurines, thiopurine *S*-methyltransferase, genotyping, malnutrition.

ANOTĀCIJA

Pasaulē pieaug pacientu skaits ar iekaisīgām zarnu slimībām (IZS), un Latvija nav izņēmums. IZS ir kļuvušas par izplatītām slimībām attīstītajās valstīs, un pēdējo 50 gadu laikā saslimstība ar tām ir ievērojami palielinājusies. Tiopurīnu monitorings ir daudzsološa stratēģija, kas var optimizēt IZS terapiju. Kaut gan klīniskajā praksē arvien vairāk tiek izmantota tiopurīna metabolisma analīze, ir ierobežoti pierādījumi un trūkst vispārējas pieejas tiopurīna uzraudzībai IZS ārstēšanā. Azatioprīnatioprīnu plaši izmanto IZS ārstēšanā, tomēr nevēlamās blakusparādības ir reģistrētas aptuveni 10% pacientu, kuriem IZS ārstēšanai lietoja tiopurīnus. Enzīmam tiopurīna metiltransferāzei (TPMT) ir nozīmīga loma tiopurīnu metabolismā, un zemā TPMT aktivitāte ir saistīta ar izmainītu tiopurīna metabolismu, citotoksisko metabolītu pārprodukciju un mielosupresiju. Pacientiem ar IZS TPMT genotipēšanu var veikt pirms ārstēšanas, lai novērtētu ārstēšanas riskus, tomēr tas neizslēdz iespēju, ka pacienti var nepanest tiopurīnus.

Promocijas darba mērķis bija pacientiem ar iekaisīgām zarnu slimībām izpētīt tiopurīna metabolisma individuālas izmaiņas, izmantojot imunoloģiskās un molekulārās bioloģijas metodes, un izvērtēt pacientu vielmaiņas stāvokli.

Tajā analizēta TPMT genotipa un fenotipa noteikšana pacientiem ar IZS, kā arī tiek identificēti malnutrīcijas parametri, kas ir saistīti ar izteiktām vielmaiņas stāvokļa izmaiņām un ir slimības aktivitātes indikatori.

Pētījuma rezultāti parādīja, ka izplatīto TPMT alēļu biežums Latvijas populācijā, kas sirgst ar IZS, bija līdzīgs citām Eiropas populācijām. Homozigotais savvaļas tipa TPMT *1/*1 genotips bija visizplatītākais genotips pacientiem ar čūlaino kolītu un Krona slimību, un TPMT *3A bija visizplatītākais polimorfisms.

Pacientiem ar augstāku IZS risku būtu ieteicams noteikt prioritāti TPMT genotipēšanai vai fenotipēšanai, lai palīdzētu prognozēt tiopurīna izraisītas zāļu blakusparādības un noteiktu personalizētas terapijas iespējas. Pacientiem ar IZS un augstu slimības aktivitātes indeksu bija ievērojami palielināts malnutrīcijas risks, savukārt pacientiem ar Krona slimību tika konstatēta muskuļu masas samazināšanās, ko var izmantot kā slimības aktivitātes paredzamo indikatoru.

Atslēgvārdi: iekaisīgas zarnu slimības, tiopurīni, tiopurīna S-metiltransferāze, genotipēšana, malnutrīcija.

TABLE OF CONTENTS

ANNOTATION 3
ANOTĀCIJA 4
ABBREVIATIONS 7
INTRODUCTION 9
Topicality, novelty, and practical implications of the study 9
Aim of the study 10
Tasks of the study 10
Hypothesis of the study 10
1. LITERATURE OVERVIEW 11
1.1. Inflammatory bowel disease 11
1.1.1. IBD definition and characteristics 11
1.1.2. IBD epidemiology 11
1.1.3. IBD pathophysiology 12
1.1.4. IBD classification, disease activity and staging 13
1.2. Thiopurine group medications 17
1.2.1. Molecular structure and metabolism 17
1.2.2. Thiopurine side effects 18
1.3. Thiopurine S-methyltransferase 19
1.3.1. TPMT genotype 20
1.3.2. TPMT phenotyping 22
1.3.3. TPMT genotyping versus phenotyping 22
1.4. Treatment of IBD and therapeutic drug monitoring 23
1.5. Malnutrition in IBD patients 25
2. MATERIALS AND METHODS 29
2.1. Patient selection 29
2.2. Patient data and information acquisition 29
2.3. Blood samples preparation 31
2.4. TPMT genotype detection 31
2.5 TPMT phenotype detection 31
2.6. Bioelectrical impedance analysis method 32
2.7. Control group 32
2.8. Statistical analysis 32
3. RESULTS 34
3.1. TPMT genotyping (First study) 34

3.1.1. Study participants 34
3.1.2. TPMT genotyping 35
3.1.3. Association of TPMT polymorphisms with different clinical factors 37
3.2. Method of detection of thiopurine methyltransferase polymorphisms 38
3.2.1. Optimising the method of TPMT genotype detection 38
3.2.2. Characterisation of probes and primers used in our protocol 39
3.2.3. The patented method of TPMT testing 43
3.2.3.1. Extraction and purification of DNA 44
3.2.3.2. Evaluation of the TPMT testing qPCR method 44
3.2.3.3. External Quality Assessment 44
3.2.4. Comparison with alternative PCR methods 45
*3.2.4.1. Detection of TPMT *2* 45
*3.2.4.2. Detection of TPMT *3B and TPMT *3C* 47
3.3. TPMT phenotyping (Second study) 50
3.4. IBD malnutrition (Third study) 52
3.4.1. Patient study group 52
3.4.2. Nutritional screening 54
3.4.3. Bioelectrical impedance analysis 57
DISCUSSION 61
CONCLUSIONS 68
PRACTICAL RECOMMENDATIONS 69
PUBLICATIONS AND THESIS OF THE AUTHOR 70
ACKNOWLEDGEMENTS 75
REFERENCES 77
SUPPLEMENTS 87
Publication I 87
Publication II 95
Publication III 99
Publication IV 107
Latvian patent Nr. LV 15508 B1 112
Central medicine ethical permission 121
Genome research council permission 122
Pauls Stradiņš Clinical University Hospital Ethical permission 123

ABBREVIATIONS

ADR – adverse drug reaction

AZA – azathioprine

BIA – bioelectrical impedance analysis

BMI – body mass index

CD – Crohn's disease

CDAI – Crohn's disease activity index

CPIC – Clinical Pharmacogenetics Implementation Consortium

DNA – deoxyribonucleic acid

ECCO – European Crohn's and Colitis Organisation

ELISA – enzyme-linked immunosorbent assay

ESGAR – European Society Gastrointestinal and Abdominal Radiology

ESPEN – European Society for Clinical Nutrition and Metabolism

EQA – external quality assessment

FDA – Food and Drug Administration

FFM – fat-free mass

IBD – inflammatory bowel diseases

IVD – in vitro diagnostic

HPLC – high-performance liquid chromatography

HPRT – hypoxanthine phosphoribosyltransferase

MBF – metabolic body fat

meTIMP – methylthioinosine monophosphate

meMP – methyl-mercaptopurine

meTG – methyl-thioguanine

meTGNs – methyl-thioguanine nucleotides

MMP – methylmercaptopurine

MP – mercaptopurine

MUST – Malnutrition Universal Screening Tool

NRS2002 – Nutritional Risk Screening Score 2002

PBF – percent body fat

PCR – polymerase chain reaction

qPCR – quantitative polymerase chain reaction

RFLP – restriction fragment length polymorphism

SLM – soft lean mass

SNP – single nucleotide polymorphism

SAG – subjective global assessment

TBW – total body water

TG – thioguanine

TGNs – thioguanine nucleotides

TNF – tumour necrosis factor

TPMT – thiopurine methyltransferase

UC – ulcerative colitis

XO – xanthine oxidase

INTRODUCTION

Topicality, novelty, and practical implications of the study

Thiopurine monitoring is a guideline for future medical treatment in autoimmune diseases. It considers the interindividual variability of pharmacokinetics and thus enables personalised pharmacotherapy. Individual thiopurine metabolism analysis affects treatment outcomes in patients with inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), as well as therapy efficiency and drug toxicity (Di Paolo and Luci, 2021). Thiopurine methyltransferase (TPMT) is an essential enzyme for biotransformation and its determination is an important part of therapy before administering thiopurines (Marinaki and Arenas-Hernandez, 2020). Histological remission is the main aim that should be achieved in IBD patient treatment. However, studies show that the histological remission of patients is still difficult to achieve, despite the use of modern treatment protocols.

Thiopurine monitoring is a promising strategy that can optimise IBD therapeutics. Despite the increasing adoption of thiopurine metabolism analysis in clinical practice, there is limited data of evidence and a lack of an overall approach to thiopurine monitoring in the management of IBD. Thiopurine methyltransferase (TPMT) and inter-individual variability of TPMT genetic polymorphisms influences drug metabolism and concentration of drug metabolites, 6-thioguanine (TG) and 6-methylmercaptopurine (MMP), which have been variably associated with therapy efficiency and thiopurine toxicity as induced life-threatening adverse effects (Dickson et al., 2021; Warner et al., 2018). The study has significant scientific and practical applications. So far, neither TPMT expression, nor TPMT activity, nor TPMT polymorphism in patients who initiate thiopurine therapy are identified in Latvia. It is also a novelty in European countries. The need for individual thiopurine metabolism analysis is very high, as patients with TPMT deficiency may be at increased risk of serious health problems when using thiopurine medications.

Individual therapeutic drug monitoring by analysing the metabolism of the thiopurines may significantly affect the treatment outcomes in patients with IBD, reducing the risk of side effects, drug overdosing and associated maintenance costs in patients with TPMT deficiency. The hyperactivity of the TPMT enzyme means that the therapeutic effect will not be achieved and high doses of thiopurines can lead to hepatotoxicity (Feuerstein et al., 2017; Warner et al., 2018).

Testing the TPMT phenotype or genotype before initiating thiopurine therapy is the safest way to determine the probability of developing harmful side effects with the use of this drug (Lim and Chua, 2018). The developed methodology will be implemented in clinical practice.

The widespread involvement of gastrointestinal tract disorders raises particular attention to the nutritional requirements of IBD patients. Several factors can affect nutritional status and promote the

development of malnutrition, such as the duration and activity of the disease. Other components that influence the development of malnutrition include increased energy requirements, reduced nutritional uptake, reduced breakdown and absorption of nutrients, and malabsorption. Malnutrition is associated with negative clinical outcomes and higher rates of IBD mortality. Malnourished patients are more likely to undergo repeated admissions within 15-day periods and higher mortality rates in three years. Patients are at increased risk of complications, which therefore increases the length of hospital stays and treatment costs (Landi, 2019).

Aim of the study

The aim of the study was to investigate the interindividual variability of thiopurine metabolism using immunological and molecular biology methods with metabolic status in patients with inflammatory bowel disease.

Tasks of the study

1. To determine TPMT genotype in IBD patients using molecular biology methods (Study 1).
2. To perform individual therapeutic drug monitoring of thiopurine metabolism of the TPMT phenotype using the enzyme-linked immunosorbent assay (ELISA) method (Study 2).
3. To identify malnutrition parameters associated with metabolic status in IBD patients (i.e. classified as by low and high clinical activity) as an anticipatory indicator of disease activity (Study 3).

Hypothesis of the study

Thiopurine metabolism data will confirm the diversity of thiopurine phenotypes in the Latvia IBD population, and IBD activity is affected by individual metabolic status.

1. LITERATURE OVERVIEW

1.1. Inflammatory bowel disease

1.1.1. IBD definition and characteristics

IBD is characterised as a chronic, multifactorial, autoimmune disease. The most common types of IBD are UC and CD, two idiopathic intestinal diseases that are differentiated by location and depth of involvement of the bowel wall. UC is characterised by inflammation of the colonic mucosa, whereas CD can affect any part of the gastrointestinal tract, but most frequently affects the terminal ileum and colon, and results in transmural ulceration (Colombel et al., 2019). IBD is characterised by cycles of remission and relapse, with complex interactions among genetics, environmental factors, and the immune system (Ananthakrishnan, 2015). UC and CD can cause autoimmune disorders in other organ systems. The European Crohn's and Colitis Organisation (ECCO) - European Society Gastrointestinal and Abdominal Radiology (ESGAR) Guidelines for diagnostic assessment in IBD says that single reference standards for the diagnosis of CD or UC do not exist. The diagnosis of CD or UC is based on combinations of clinical, biochemical, stool, endoscopic, cross-sectional imaging, and histological investigations (Maaser, 2019).

1.1.2. IBD epidemiology

The number of patients with IBD is increasing worldwide, and Latvia is no exception. IBD has become a prevalent disease in industrialised countries with the incidence rising significantly over the past 50 years (Levine, 2020). The rate of IBD is much higher in North America and Europe than in Asia or Africa. The North American incidence is from 2.2–19.2 cases per 100 000 person-years for UC and 3.1–20.2 cases per 200 000 person-years for CD. Diagnosed cases of UC and CD in the United States are 238 per 100 000 and 201 per 100 000 populations, respectively (Su et al., 2019). The highest reported prevalence values were in Europe, accounting for 505 UC cases per 100 000 in Norway and 322 CD cases per 100 000 person-years in Germany (Ng et al., 2017). Although most IBD occurs in individuals aged 15 to 30, up to 25% of patients will develop IBD during adolescence and the second peak of 10–15% develop IBD after age 60. CD is slightly more common in females than males, despite UC having similar rates in both genders (Su et al., 2019).

1.1.3. IBD pathophysiology

The pathogenesis of both UC and CD is still not fully established. The hypothesis is that they start from inappropriate activation of the mucosal immune system in response to the microbiome in a genetically susceptible host (Spekhorst et al., 2014).

In healthy people, the lamina propria regularly contains a differing cluster of immune cells and secreted cytokines. Cytokines incorporate anti-inflammatory mediators that down-regulate immune responses, as well as pro-inflammatory mediators from both innate and adaptive immune cells that restrain intemperate sections of intestinal microbiota and guard against pathogens. Noninflammatory guards, such as phagocytosis by macrophages, likely help guard against microbes entering the lamina propria and minimise tissue damage. A homeostatic adjustment is maintained between regulatory T cells and effector T cells (Th1, Th2 and Th17). A comparison between healthy patients and patients with intestinal inflammation is shown in Figure 1. In case of inflammation, a few occasions contribute to the expanded bacterial introduction, including a disturbance of the mucus layer, dysregulation of epithelial tight intersections, expanded intestinal permeability, and increased bacterial adherence to epithelial cells. In IBD, innate cells produce expanded levels of tumour necrosis factor (TNF) alpha, interleukin-1 beta, interleukin-6, interleukin-12, and interleukin-23. The development of the lamina propria is checked with ongoing expanded numbers of CD4+ T cells, particularly pro-inflammatory T-cell subgroups. Increased production of chemokines comes from the enlistment of extra leukocytes during cycles of inflammation (Abraham and Cho, 2009). Fistulas, perianal disease, and colonic and small bowel obstructions are common in CD patients. Cryptitis and crypt abscesses are observed in both UC and CD, while crypt architecture is more distorted in the case of UC (Yeshi et al., 2020).

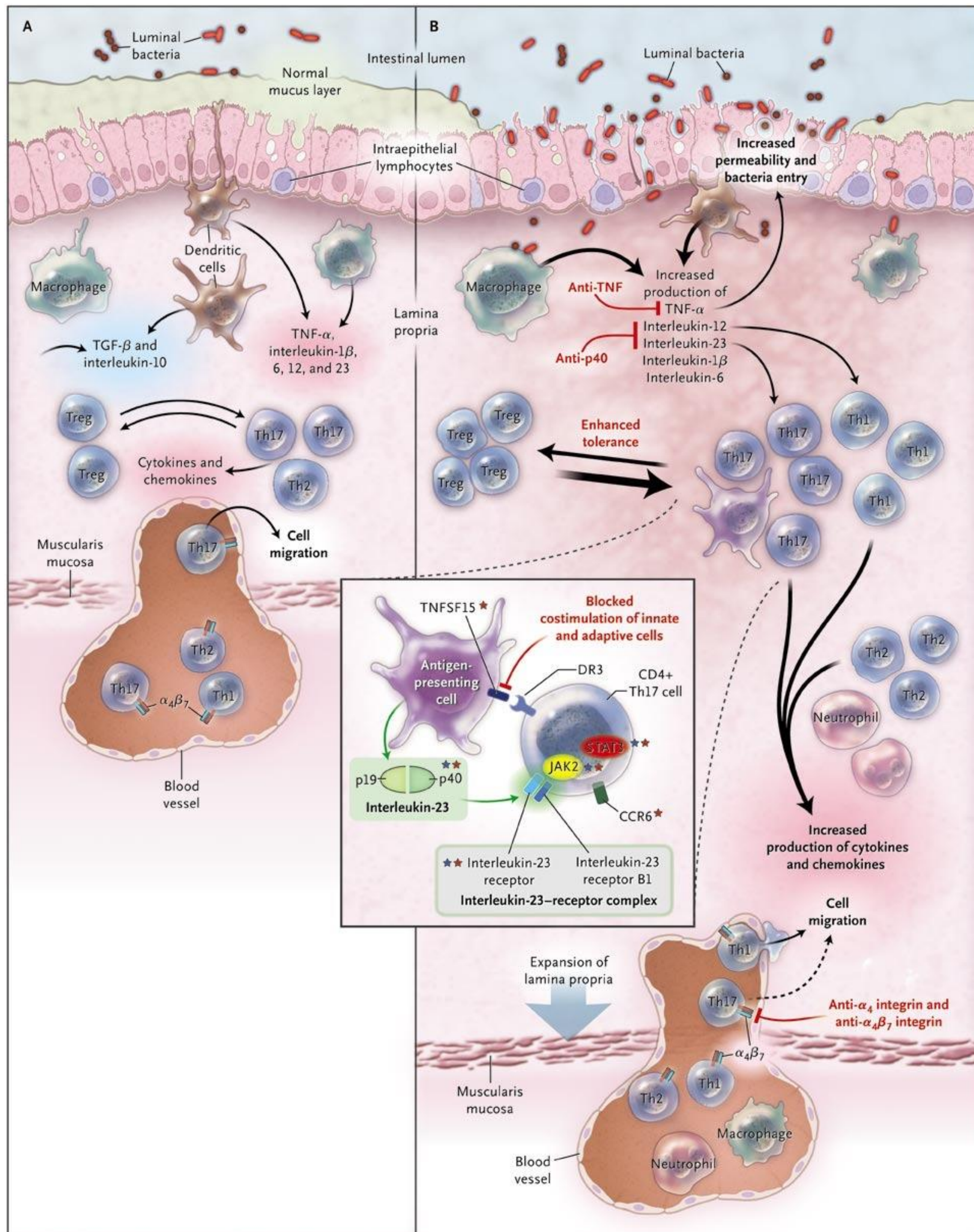


Figure 1. Scheme of an immune system of intestinal inflammation in a healthy patient (A) and a patient with an IBD (B). Adapted from (Abraham and Cho, 2009).

1.1.4. IBD classification, disease activity and staging

CD and UC show heterogeneity in many clinical features. They are differentiated by the location and nature of inflammation (Figure 2). UC causes inflammation and ulceration of the inner lining of the colon and rectum. Disease onset is between ages 30–40 years with no gender

predominance (Yeshi et al., 2020). UC can be present in the rectum and involve the left-sided colon or total/pancolitis. CD is more common in the terminal ileum part of the ileocecal region, but can also involve segmental damage of the large intestine, forming strictures or fistulas. Smoking, antibiotic use, and diet are potentially preventable risk factors for IBD (Forbes, 2016).

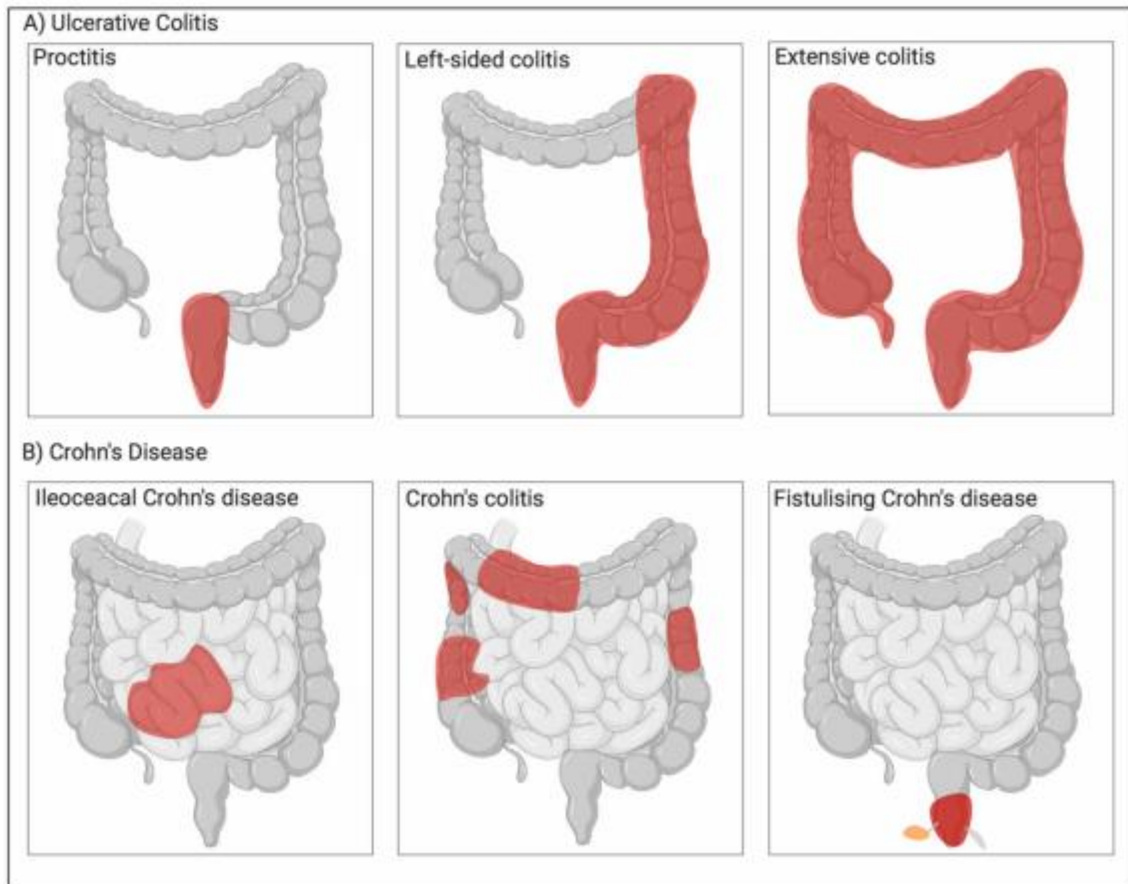


Figure 2. IBD different types. Adapted from (Yeshi et al.,2020).

In 2000, the Vienna classification was accepted, which was the first attempt to classify different clinical phenotypes of CD (Gasche et al., 2000). The Vienna Classification was followed by the Montreal classification in 2005 that describes the extent and behaviour of CD in more detail and includes a classification for UC (Silverberg et al., 2005). The Montreal classification divides CD according to the age of the patient, location, and behaviour (structuring, penetrating or non-structuring, non-penetrating) into separate emphasising perianal disease modifiers. In contrast to CD, the continuous character of damage to the mucosa in the intestine from UC makes classification simpler; the classification of UC is limited to the severity and extent (Jakubczyk et al., 2020). Both UC and CD Montreal classifications are described in Table 1.

	CD Classification		UC Classification
Age at diagnosis	A1:< 17 years A2: 17–40 years A3: > 40 years	Severity	S0: remission, no symptoms S1: mild symptoms S2: moderate symptoms S3: severe symptoms
Location, endoscopic or macroscopic estimation	L1: terminal ileal L2: colon L3: ileocolon L4: upper GI modifier: proximal disease with distal disease, such as L1 + L4, L2 + L4, L3 + L4)	Extensity	E1: ulcerative proctitis E2: left-sided UC; distal colitis E3: extensive UC, pancolitis
Behavior over time	B1: non-stricturing, non-penetrating B2: stricturing B3: penetrating P: perianal disease modifiers, such as B1p, B2p, B3p		

Table 1. The Montreal classifications for CD and UC. Adapted from (Jakubczyk et al., 2020).

For the disease activity of UC, the Mayo score is most used. The Mayo score combines endoscopic and clinical scales to assess the severity of UC. It is composed of four parts: rectal bleeding, stool frequency, physician's global assessment, and findings of flexible endoscopy (proctosigmoidoscopy or colonoscopy). Each part is rated from 0 to 3, giving a total score from 0 to 12. Within the endoscopic component of the Mayo Score, a score of 0 is given for normal mucosa or endoscopic remission UC; a score of 1 is given for mild disease with evidence of mild friability, reduced vascular pattern, and mucosal erythema; a score of 2 is indicative of moderate disease with friability, erosions, complete loss of vascular pattern, and significant erythema; and a score of 3 indicates ulceration and spontaneous bleeding (Paine, 2014). A score of 3 to 5 points in total indicates mildly active disease, a score of 6 to 10 points indicates moderately active disease, and a score of 11 to 12 points indicates severely active disease. Two compressed forms, the partial Mayo score that prohibits the endoscopy subscore and the non-invasive six-point score that comprises only the rectal bleeding and stool frequency portions, have been created and approved (Lewis et al., 2008) (Table 2).

Score	Stool frequency
0	Normal number of stools for patient
1	1 to 2 stools per day more than normal
2	3 to 4 stools more than normal
3	≥5 stools more than normal
	Rectal bleeding
0	No blood seen
1	Streaks of blood with stool less than half the time
2	Obvious blood with stool most of time
3	Blood alone passes
	Endoscopic finding
0	Normal or inactive disease
1	Mild disease
2	Moderate disease
3	Severe disease
	Physician's global assessment
0	Normal
1	Mild disease
2	Moderate disease
3	Severe disease

Table 2. Mayo scoring system for assessment of UC. Adapted from (Schroeder, 1987).

For determining CD activity, the CD activity index (CDAI) is most used. It is based on eight clinical variables, three derived from a 1-week patient diary. Each independent variable is coded so that 0 corresponds to good health and increasing positive values correspond to greater degrees of sickness. A score of less than 150 corresponds to relative disease quiescence (remission); 150–219, a mildly active disease; 220–450, a moderately active disease; and greater than 450, severe disease (Best, 2006) (Table 3).

Item(day)	Weight
No. liquid or very soft stools(each day for 7days)	×2
Abdominal pain, sum of 7 d rating (0=none,1=mild,2=moderate,3=severe)	×5
General well being (1-4)	×7
Exteraintestinal (1 per finding) Arthritis/arthralgia Mucocutaneous lesion Iritis/uveitis Anal disease (fissure, fistula,etc) External fistula	×20
Fever>36.8	
Antidiarrheal use	×30
Abdomial mass(none-0,equivocal-2,definite-5	×10
Hematocrit (males-47) (Females-42)	×6
Bodyweight (1-body weight/standard weight) ×100	×1
Total CDAI Score	

Table 3. CD activity index. Adapted from (Best, 2006).

1.2. Thiopurine group medications

1.2.1. Molecular structure and metabolism

Azathioprine (AZA) is a derivative of 6-mercaptopurine (MP) or a derivative of the 6-MP imidazole group and is a widely used thiopurine class drug for the treatment of both steroid-dependent and steroid-resistant IBD (Benmassaoud et al., 2016; Liu et al., 2015). The metabolism of thiopurines in humans has not been fully established, but it is known that four to five weeks is required before anti-inflammatory effects are seen. Severe life-threatening bone marrow toxicity can result from an overproduction of AZA metabolites such as thioguanine nucleotide (TGN) (Skrzypczak-Zielinksa, 2016; Armstrong, 2001). The metabolism of thiopurines is complex. AZA is metabolised by the liver and rapidly converted to 6-MP in the presence of sulfhydryl compounds such as cysteine and glutathione. Metabolism activates different enzymatic pathways producing active, inactive, and potentially toxic metabolites. After being absorbed from the gastrointestinal tract, 88% of AZA is converted to MP in red blood cells (Yarur et al., 2014). 6-MP is then converted to its metabolites by an intracellular multienzymatic process by three enzymes, hypoxanthine phosphoribosyltransferase (HPRT), TPMT, and xanthine oxidase (XO) (Carvalho, 2014; Lennard, 2014).

The metabolite 6-TG accounts for most of the therapeutic effects of AZA (Fangbin et al., 2016; Yarur et al. 2014). At the same time, the accumulation of 6-TG causes AZA-related side effects: the incorporation of 6-TG into deoxyribonucleic acid (DNA) induces delayed cytotoxicity and may lead to apoptotic cell death by inhibiting intracellular signalling pathways (Lennard, 2014). TG and MP can be converted by HPRT to TGNs metabolites and MP can be converted to methylthioinosine monophosphate (meTIMP) by TPMT. However, thioguanine bypasses the conversion to this metabolite. Thiopurines can be converted to inactive metabolites such as methyl-mercaptopurine (meMP), methyl-thioguanine (meTG), and methyl-thioguanine nucleotides (meTGNs) by TPMT (Hosni-Ahmed, 2011) (Figure 3). The enzyme TPMT plays an important role in determining the number of cytotoxic 6-TGNs.

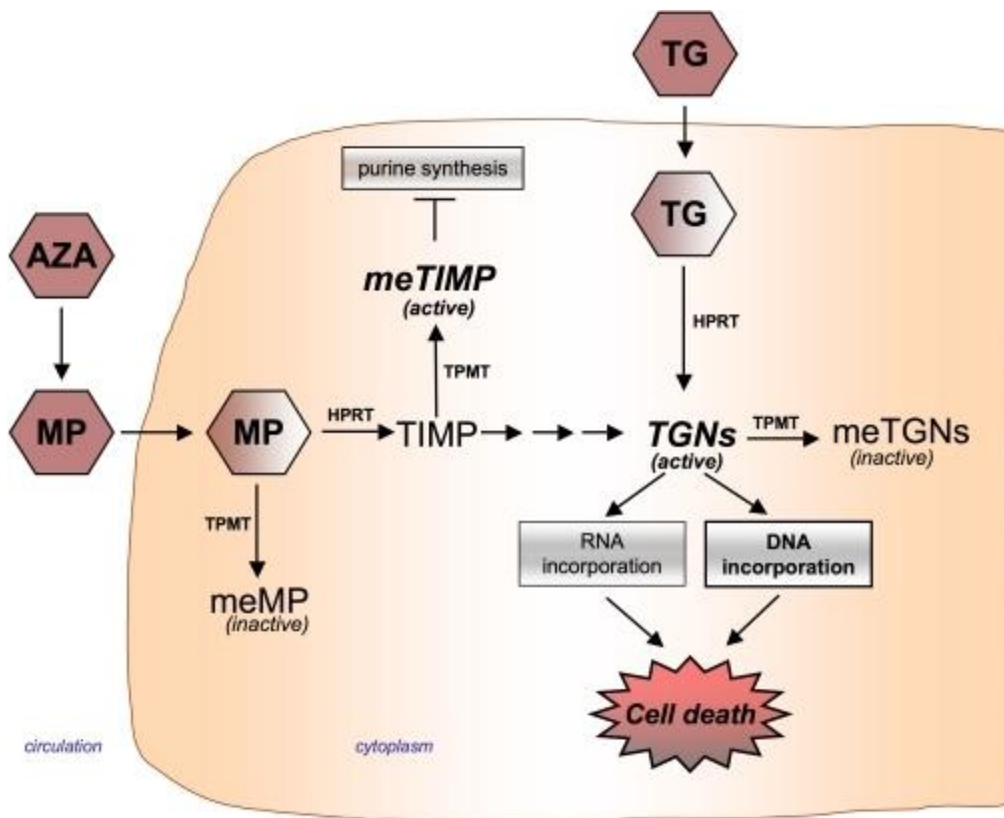


Figure 3. Thiopurine drug metabolism pathway. Adapted from (Hosni-Ahmed A et al., 2011).

1.2.2. Thiopurine side effects

Genetic polymorphisms in TPMT affect the activity of this enzyme and may lead to the toxicity of thiopurine drugs, which can cause life-threatening side effects.

Adverse reactions occur in approximately 10% of patients with IBD treated with AZA and approximately 10–20% of these patients discontinued treatment due to adverse reactions (Liu et al, 2015; Ardizzone et al., 2004). Most adverse reactions occur within the first three months of treatment but may occur up to 3 years after treatment (Kim and Choe, 2013; Frei et al., 2013). Complications were observed at 1 and 3 months in 26% and 93% of patients receiving the full dose of AZA (Benmassaoud et al., 2016). The most common complications of AZA are gastrointestinal disorders, hepatotoxicity, infections, and myelosuppression (Kim and Choe, 2013; Frei et al., 2013). However, other complications include pancreatitis, malignancies, and allergic skin reactions. Patients receiving the full dose of AZA are particularly at increased risk of developing lymphoma and skin malignancies. Mercaptopurine is reported to be better tolerated by about 50% of patients intolerant to AZA (Frei et al., 2013).

Liu et al. (2015) conducted a meta-analysis of 11 studies and found that TPMT genetic polymorphism was more associated with myelosuppression and AST disorders than with hepatotoxicity. Myelosuppression is a dose-dependent side effect of thiopurines, occurring in approximately 2–5% of European patients. It can develop at any time, but in 25% of cases, it occurs after the first year (Frei et al., 2013). Early myelosuppression can be avoided by measuring TPMT activity prior to thiopurine administration, but each patient will still require regular haematological monitoring of the blood count (Liu et al. 2015).

Infectious complications during thiopurine therapy may occur even in the absence of dose-dependent leukopenia, especially when thiopurine is used in combination with corticosteroids, which may lead to dose-dependent lymphocyte depletion (Frei et al., 2013).

After a few years of treatment, hepatotoxicity may manifest as an early indicator of drug-induced hepatitis as nodular regenerative hyperplasia or fibrosis (Frei et al., 2013). Thiopurine-induced hepatotoxicity is more likely to be dose-dependent and in many patients, elevated transaminases respond to dose reduction of thiopurines (Liu et al. 2015).

The most common specific adverse reactions are nausea and vomiting, which are present in up to 15% of patients (Ribaldone et al, 2019; Tripathi and Feuerstein, 2019). Several experts have recommended slowly increasing the dose when starting thiopurine therapy or taking it before sleeping. Other common side effects include headache, fatigue, weakness, weight loss, stomatitis, alopecia, arthralgia, muscle weakness, and rash, which can occur in more than 10% of patients. If these side effects occur, it should be determined whether they disappear after dose reduction. In the case of arthralgia and myalgia, transitioning from AZA to mercaptopurine should be considered (Frei et al., 2013; Asadoy et al., 2017).

Pancreatitis is also a serious side effect and occurs in up to 4% of patients, especially in the first weeks of treatment (Frei et al., 2013). A small and asymptomatic increase in serum amylase is common and some experts recommend reducing the dose or stopping treatment if this occurs. If the increase in amylase is associated with typical pain symptoms (toxic pancreatitis), thiopurines should be discontinued. Switching to mercaptopurine after AZA-induced pancreatitis is not recommended as these patients are less likely to tolerate it well (Frei et al., 2013; Ribaldone et al, 2019).

1.3. Thiopurine S-methyltransferase

TPMT is a cytoplasmic enzyme encoded by the TPMT gene on the short arm of chromosome 6 (6p22.3) (Coelho et al., 2016; Asadoy et al., 2017). The TPMT enzyme catalyses the S-methylation process in the body and metabolises cytostatic drugs in the inactive methylated form in patients taking thiopurine drugs (Lennard, 2014). Despite the pharmacological role of TPMT in metabolism, the metabolism of thiopurines in the body has not been fully studied (Gonzalez-Lama and Gisbert, 2016).

Various clinical guidelines recommend that TPMT be determined prior to initiate thiopurine therapy (Benmassauiud et al., 2016; Liu et al., 2015; Lennard, 2014; Liu et al., 2016). This can be achieved by two methods: 1) evaluating TPMT enzyme activity in circulating red blood cells (RBCs) showing the TPMT phenotype or 2) identifying the TPMT variant genotype associated with enzyme deficiency using PCR (Roy et al., 2016; Goel et al., 2015). Thus, TPMT enzyme activity is determined by biochemical methods and polymorphisms affecting TPMT activity are determined by molecular biology methods.

1.3.1 TPMT genotype

From the literature, around 85–95% of people have two functioning TPMT alleles, it is a wild-type genotype associated with normal TPMT enzyme activity. In total, about 40 TPMT gene polymorphism variants associated with decreased TPMT activity have been described. TPMT*1 is a functioning or normal activity allele (Asadov et al., 2017) (Figure 4).

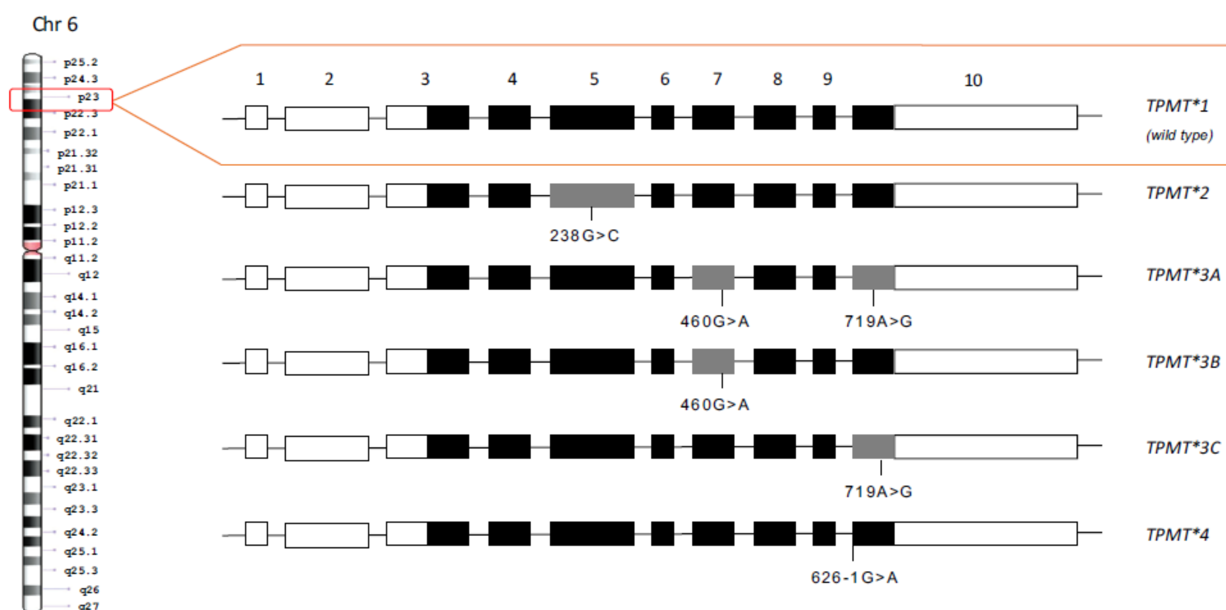


Figure 4. TPMT gene and common mutant alleles. Adapted from (Asadov et al., 2017).

There are three main models of TPMT enzyme activity: 1) homozygous patients with two non-functional alleles and low enzyme activity; 2) heterozygous individuals with one non-functional and one functional allele and moderate TPMT activity; and 3) homozygous wild-type individuals with two functioning TPMT gene alleles and normal or high TPMT activity (Lennard, 2014; Dean, 2012; Chouchana et al., 2014) (Figure 5).

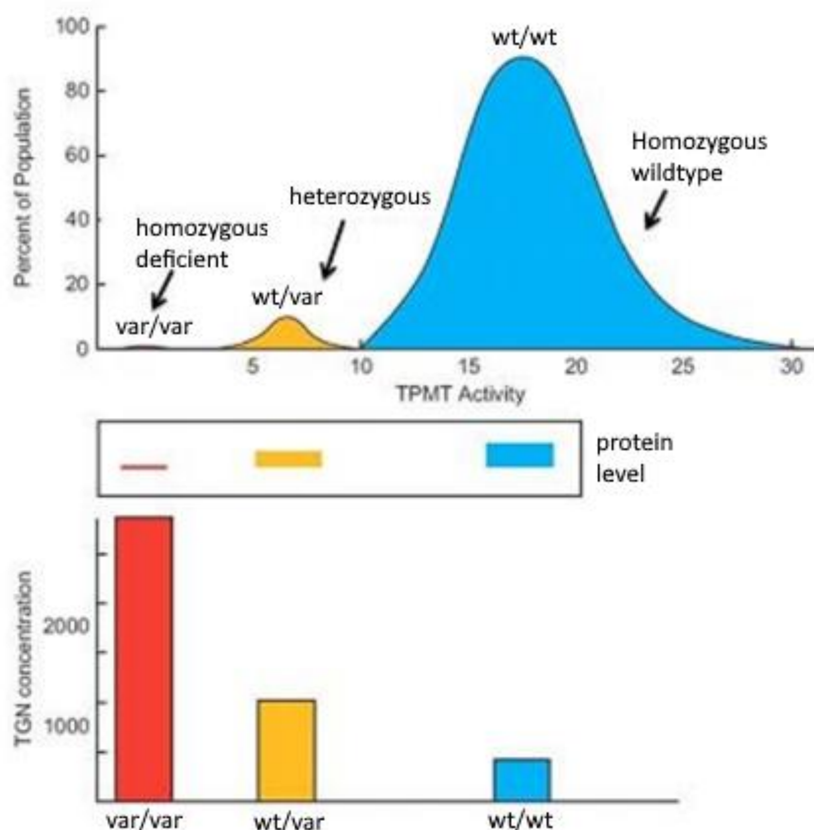


Figure 5. TPMT enzyme activity depending on TPMT genotype and TGN metabolite levels. Adapted from (Relling et al., 2019).

TPMT polymorphisms differ between various ethnic groups. The most common alleles in Europeans that reduce TPMT activity are TPMT * 2 (c.238G> C), TPMT * 3A (c.460G> A and c.719A> G), TPMT * 3B (c.460G> A), and TPMT * 3C (c.719A> G).

TPMT enzyme activity is associated with single nucleotide polymorphisms (SNPs), TPMT * 2 is associated with rs1800462, TPMT * 3B with rs1800460, and TPMT * 3C with rs1142345. However, TPMT * 3A is associated with both rs1800460 and rs1142345 (Lennard 2014; Asadoy et al., 2017). These alleles are thought to account for 80–95% of the reduced TPMT enzyme activity. For this reason, these four alleles are used in most genotyping tests (Asadoy et al., 2017; Dean, 2012).

The frequency of mutant alleles varies between ethnic groups, but in general, the most common allele in most populations is TPMT * 3A, followed by TPMT * 3C (Carvalho et al., 2014; Fangbin et al. 2016; Almoguera et al. 2014). There are reports that Europeans have one of the highest frequencies of the TPMT * 3A genotype. In contrast, Asians and Africans have higher frequencies of the TPMT * 3C genotype (Asadov et al., 2017; Almoguera et al. 2014). TPMT * 3C is also almost the only mutant allele observed in Asians. The less common allele TPMT * 2 is found mainly in ethnic groups in South America and the Middle East, especially in Iran. The TPMT * 3B allele is rare. It is usually present in tight linkage with the *3C SNP, resulting in a common allele, *3A (Dean, 2012; Wang et al., 2010).

On average, 1 in 300 individuals lack TPMT activity and approximately 11% of people have a heterozygous allelic variant, indicating lower enzyme activity. Low TPMT enzyme activity is associated with abnormal metabolism of thiopurine drugs, overproduction of cytotoxic metabolites, and the potential for myelosuppressive effects on hematopoietic cells and adverse reactions unrelated to the pharmacological properties or dose of the substances. Bone marrow toxicity in patients with low TPMT activity receiving thiopurine at doses of 1.5–2.5 mg/kg is associated with TPMT deficiency, whereas patients with TPMT mutations in a heterozygous state are at increased risk of myelosuppression (Dean, 2012; Broekman et al., 2017; Gonzalez-Lama and Gisbert, 2016).

1.3.2. TPMT phenotyping

High-performance liquid chromatography (HPLC) with mass spectrometry, which measures TPMT activity in erythrocytes using the substrate 6-mercaptopurine (6-MP), is the most common method for determining or phenotyping TPMT enzyme activity (Coenen et al., 2015). Recent studies suggest that the HPLC method could also be used to measure TPMT activity in whole blood. These methods are less time-consuming and are less likely to make a mistake than HPLC of erythrocytes, as laborious erythrocyte washing steps required for erythrocyte isolation may be omitted. Recent studies have optimised this method and they are widely used in research (Coenen et al., 2015).

1.3.3. TPMT genotyping versus phenotyping

TPMT genotyping is highly sensitive compared to determining enzyme activity (Almoguera et al., 2014; Genneo et al., 2019). Studies have shown that the approximate sensitivity and specificity of TPMT genotyping are 88.9% (81.6–97.5%) and 99.2% (98.4–99.9%), respectively, while the approximate sensitivity and specificity of TPMT phenotyping are 91.3% (86.4–95.5%) and 92.6% (86.5–96.6%) respectively (Genneo et al., 2019).

When interpreting the performance of the TPMT enzyme, it should be considered that this diversity in enzyme activity depends on many factors, such as age, gender, ethnicity, erythrocyte mass transfusion, leukaemia and drug therapy, and drug interactions (Asadoy et al., 2017; Chouchana et al., 2014). In addition, reassessments may be required to determine TPMT activity, as treatment with AZA may result in increased TPMT activity. Therefore, determination of TPMT enzyme activity is recommended before the start of thiopurine therapy rather than during. Similarly, TPMT enzyme activity may be affected by other drugs, for example, 5-aminosalicylate drugs can reversibly inhibit TPMT activity (De Boer et al., 2007; Gilissen et al., 2005). TPMT enzyme activity may also depend on external factors such as incubation temperature, substrate source, and concentration, whereas DNA

is much more stable in genotyping. These factors should be considered before deciding on the method of determining TPMT status.

Therefore, for many reasons, TPMT genotyping is considered to be the more accurate and reliable method. In many studies, the overall agreement between TPMT genotype and phenotype is 90–95%. Although some studies have found that genotype matching was approximately 60–70% in patients with low TPMT activity, the genotype-phenotype correspondence was higher in the heterozygous group, at approximately 70–86% (Lennard, 2014; Assadoy et al., 2017).

1.4. Treatment of IBD and therapeutic drug monitoring

AZA and mercaptopurine have been used for several decades to treat IBD at standard doses of 1.5–2.5 and 0.75–1.5 mg/kg, respectively. Currently, by pre-determining TPMT activity, the dosage of AZA and mercaptopurine can be adjusted individually for each patient. TPMT is required for detoxification by S-methylation (Burchard et al., 2014). Thus, if the dose of a thiopurine is not administered based on an individual's level of TPMT activity, toxicity due to TPMT deficiency may lead to discontinuation of treatment (Gisbert et al., 2006; Liu et al., 2016; Roy et al., 2016). In contrast, higher than normal TPMT activity may lead to resistance to normal doses of AZA, and higher doses may be required for effective treatment (Cuffari et al., 2004).

AZA and mercaptopurine are the most used immunosuppressive drugs in IBD patients. Recent studies suggest that AZA can control active inflammation and prevent relapses in CD and UC, as well as reduce steroid dependence and maintain remission in IBD (Carvalho et al., 2014).

Low TPMT activity is associated with altered thiopurine metabolism, overproduction of cytotoxic metabolites, and myelosuppression. Prior to administration of thiopurines to patients with IBD, TPMT should be genotyped or phenotyped to assess treatment risks and prevent adverse reactions (Warner et al., 2018).

The Clinical Pharmacogenetics Implementation Consortium (CPIC) published recommendations for AZA dosing based on TPMT genotype (Relling et al., 2019). In patients with two functionally active TPMT alleles, TPMT enzyme activity is normal or high in most cases. In these patients, the CPIC recommends starting with the standard dose of AZA and adjusting the dose based on specific conditions. In heterozygous patients, TPMT enzyme activity is moderate. These patients are at increased risk of dose-dependent AZA-induced myelosuppression. Therefore, the CPIC recommends that these patients initiate AZA therapy at 30–70% of the target dose and titrate the dose based on tolerance. In homozygous patients with two non-functional TPMT alleles and low TPMT enzyme activity, alternative treatments have been proposed (Benmassaoud et al., 2016; Dean, 2012). Alternative treatment is biological treatment such as anti-TNF therapy. Interestingly, in a separate

study, TPMT testing to determine the dose of AZA in patients with moderate or low TPMT enzyme activity reduced haematological adverse events by 89% (Feuerstein et al., 2017).

The Food and Drug Administration (FDA) guidelines issued in 2015 recommended the use of TPMT genotyping or phenotyping prior to beginning AZA treatment to allow the identification of patients at increased risk of toxicity who will require a reduced starting dose of AZA or alternative therapies. However, the FDA emphasises that the determination of TPMT prior to thiopurine therapy does not preclude patients from being monitored for blood counts during therapy (Dean, 2016; Warner et al., 2018).

Although TPMT genotyping and phenotyping can be used to identify patients at increased risk of bone marrow toxicity, monitoring of therapeutic agents is still recommended during treatment with AZA (Yarur, 2014). Standard laboratory tests consist of a complete blood count, liver transaminases, amylase, lipase, platelet count, and creatinine. Some authors recommend checking the blood count every week during the first month of AZA treatment, followed twice a month during the second and third months, and every month thereafter. Liver enzyme testing should be performed every 3 months (Gennep et al., 2019). Other authors recommend blood tests every 2 weeks for the first months. If signs of myelosuppression develop, AZA should be discontinued immediately (Warner et al., 2018).

As described in Section 1.2.1., the toxicity of thiopurines results from the metabolites 6-MMP and 6-TGN. Dose escalation to achieve therapeutic levels of 6-TGN may result in high levels of 6-MMP and an increased 6MMP / 6TGN ratio is associated with a poor therapeutic response and increased prevalence of side effects. Close monitoring of the metabolite should be considered to avoid toxicity or underdosing of thiopurines (Gonzalez-Lima and Gisbert, 2016).

The addition of allopurinol to thiopurine therapy is a possible method of improving thiopurine therapy. This combination has been shown to improve therapeutic levels of 6TGN and to reduce serum concentrations of the toxic metabolite 6MMP, although the mechanism by which this occurs is still unclear. One theory is that allopurinol directly inhibits the XO enzyme and therefore contributes to the increase in serum levels of the active 6TGN metabolites (Blaker et al., 2013). Another mechanism, possibly the addition of allopurinol, inhibits TPMT by producing 6-thioxanthine. The last proposed mechanism is that allopurinol may increase HPRT enzyme activity. This classic combination of thiopurine and allopurinol usually involves a dose reduction of thiopurines of at least 50% and the addition of 100 mg allopurinol, although recent studies suggest that lower and safer doses may also be beneficial (Gonzalez-Lima and Gisbert, 2016).

1.5. Malnutrition in IBD patients

Active IBD is correlated with the systemic response of the body's immune system, activating a hypermetabolic state and protein degradation (Argiles et al., 2015). These conditions lead to malnutrition, which significantly increases the risk of impaired clinical outcomes, such as delayed recovery and increased mortality (Landi et al., 2019; Friedman et al., 2018).

The widespread involvement of gastrointestinal tract disorders raises attention to the nutritional requirements of IBD patients (Friedman et al., 2018). Several factors can affect nutritional status and promote the development of malnutrition, such as the duration and activity of the disease. Other components that influence the development of malnutrition include increased energy requirements, reduced nutritional uptake, reduced breakdown and absorption of nutrients, and malabsorption (Bischoff et al., 2020).

Malnutrition is associated with negative clinical outcomes and higher rates of IBD mortality (Raslan et al., 2011). Malnourished patients are more likely to undergo repeated admissions within 15-day periods and higher mortality rates in three years. Malnourished patients are also at increased risk of complications, which increase the length of hospital stays and treatment costs (Lim et al, 2012). Screening in Emergency Units has shown the prevalence of nutritional risk of 35.3% and 28.5% according to the screening tools Nutritional Risk Screening Score 2002 (NRS2002) and Malnutrition Universal Screening Tool (MUST), respectively. Hence, it is important to detect undernutrition as early as possible (Raupp et al., 2018).

Several screening tools are recommended by the European Society of Clinical Nutrition and Metabolism, including the NRS2002 and MUST for nutritional assessment in hospital settings (Kondrup et al., 2003). MUST is a five-step screening tool to identify adults who are malnourished, at risk of malnutrition or undernutrition. It includes questions about body mass index (BMI), weight loss in the past 3–6 months, and nutritional intake for >5 days (Figure 6).

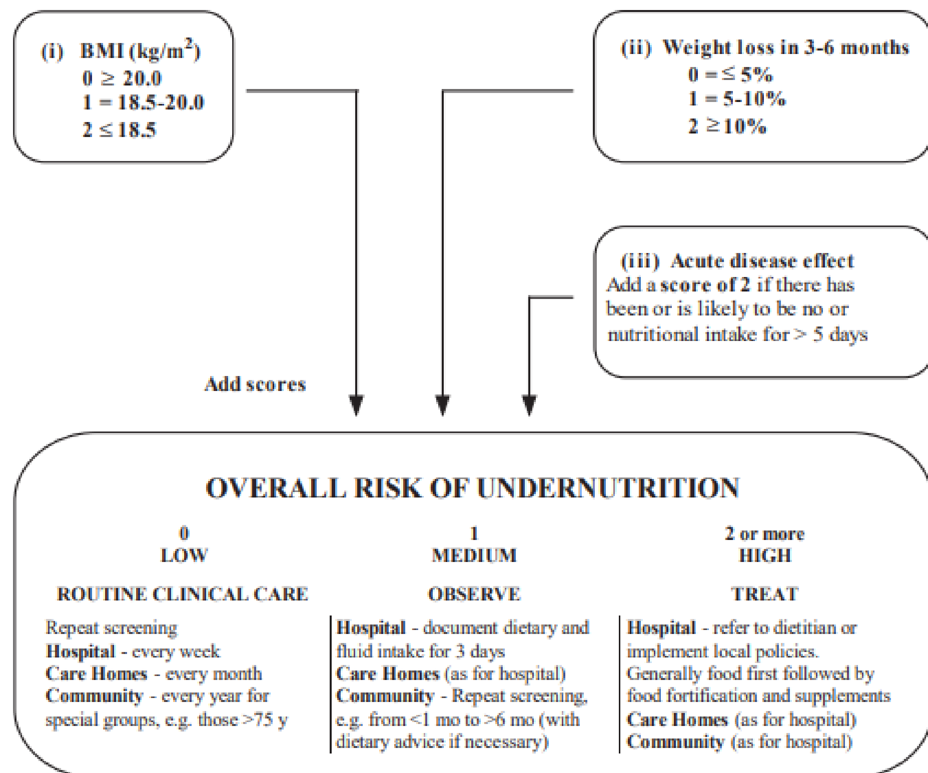


Figure 6. Malnutrition Universal Screening Tool. Adapted from (Kondrup et al., 2003).

The aim of the NRS2002 system is to detect the presence of undernutrition and the risk of developing undernutrition. NRS2002 contains the same nutritional components as MUST but also grades the severity of disease as a reflection of the increased nutritional requirements. It includes four questions for pre-screening for departments with few at-risk patients. If one of the answers is positive, then the full screening should be completed. A total score of ≥ 3 indicates that a patient is ‘at nutritional risk’ (Figure 7).

Nutritional Risk Screening (NRS 2002)

Table 1 Initial screening			
		Yes	No
1	Is BMI <20.5?		
2	Has the patient lost weight within the last 3 months?		
3	Has the patient had a reduced dietary intake in the last week?		
4	Is the patient severely ill ? (e.g. in intensive therapy)		
Yes: If the answer is 'Yes' to any question, the screening in Table 2 is performed. **No**: If the answer is 'No' to all questions, the patient is re-screened at weekly intervals. If the patient e.g. is scheduled for a major operation, a preventive nutritional care plan is considered to avoid the associated risk status.			

Table 2 Final screening			
Impaired nutritional status		**Severity of disease (≈ increase in requirements)**	
Absent **Score 0**	Normal nutritional status	Absent **Score 0**	Normal nutritional requirements
Mild **Score 1**	Wt loss >5% in 3 mths or Food intake below 50–75% of normal requirement in preceding week	Mild **Score 1**	Hip fracture* Chronic patients, in particular with acute complications: cirrhosis*, COPD*. *Chronic hemodialysis, diabetes, oncology*
Moderate **Score 2**	Wt loss >5% in 2 mths or BMI 18.5 – 20.5 + impaired general condition or Food intake 25–60% of normal requirement in preceding week	Moderate **Score 2**	Major abdominal surgery* Stroke* *Severe pneumonia, hematologic malignancy*
Severe **Score 3**	Wt loss >5% in 1 mth (>15% in 3 mths) or BMI <18.5 + impaired general condition or Food intake 0-25% of normal requirement in preceding week in preceding week.	Severe **Score 3**	Head injury* Bone marrow transplantation* *Intensive care patients (APACHE>10).*
Score:	+	**Score:**	**= Total score**
Age	if ≥70 years: add 1 to total score above	**= age-adjusted total score**	
Score ≥3: the patient is nutritionally at-risk and a nutritional care plan is initiated **Score <3**: weekly rescreening of the patient. If the patient e.g. is scheduled for a major operation, a preventive nutritional care plan is considered to avoid the associated risk status.			

Figure 7. Nutritional Risk Screening. Adapted from (Kondrup et al., 2003).

As reported by a review of 83 studies, both screening tool performances were rated fair to well for predicting clinical outcomes in adult patients (Van Bokhorst-de van der Schueren, 2014). The identification of sensitive new metabolic markers for the early diagnosis of IBD-induced malnutrition will be a future challenge for targeted IBD care.

CD leads to malnutrition in approximately 65–75% of patients (Scaldaferri, 2017) and specific nutritional deficiencies, that may be caused by low dietary intake, changes in metabolism, increased intestinal protein loss, and nutrient malabsorption (Jahnsen, 2003; Wędrychowicz, 2016). The nutritional status of IBD patients is frequently altered, even when the disease is in remission, although it is directly related to the severity of the disease (Casanova, 2017; Back, 2017).

Malnutrition is an objective disease activity parameter for patients with IBD, particularly CD, and is an indicator of systemic damage or inflammatory activity. Active disease is correlated with the systemic response of the body's immune system, activating a hypermetabolic state and protein degradation, decreasing protein synthesis (Argiles, 2015). These conditions lead to malnutrition, which significantly increases the risk of impaired clinical outcomes, such as delayed recovery or increased

mortality (Landi, 2019). Inadequate body composition and malnutrition have been associated with poor outcomes, such as a higher frequency of postoperative complications, longer hospital stays, decreased quality of life, and higher health costs (Casanova, 2017). The severity of malnutrition depends on the activity, duration and extent of the disease, and inflammatory response which drives catabolism (Forbes, 2016).

To evaluate malnutrition, basic anthropometry techniques were used, such as BMI and biochemical parameters; however, these are not accurate enough to estimate body composition. In a high proportion of IBD patients, these values may be within normal ranges despite having altered body composition, therefore bioelectrical impedance analysis (BIA) is used to calculate total body water (TBW) and to estimate fat-free mass (FFM) (lean mass) and muscle and fat mass (Casanova, 2017). BIA is easy, non-invasive, relatively inexpensive and can be performed in almost any patient because it is portable (Kyle, 2004). It is recommended that screening should be performed within the first 24–48 h after first contact and at regular intervals thereafter. For those identified as being at risk, a nutritional screening nutritional assessment should be conducted (Cederholm, 2017).

Recent studies have shown that between 22% and 60% of patients with IBD have FFM depletion. This is significant because FFM depletion is associated with negative outcomes including major postoperative complications, small bowel resection, primary non-response to anti-TNF agents, and osteopenia. Traditional nutritional measurements such as BMI correlate poorly with indices of FFM in patients with CD, resulting in an increased risk for under-recognition and underestimation of the extent of nutrition depletion when relying on these (Wood, 2020). Reduced muscle mass has been included in the Global Consensus for Diagnosing Malnutrition in Adult Patients (Cedeholm, 2019).

2. MATERIALS AND METHODS

2.1. Patient selection

This dissertation was a prospective, comparative group multicentre study including patients from Pauls Stradiņš Clinical University Hospital (PSCUH) Gastroenterology, Hepatology and Nutrition Therapy Center, in collaboration with the Latvian University, Latvian Biomedical Research and Study Center. The study was planned to include 244 patients of both sexes with IBD (UC or CD).

Patient inclusion criteria were morphologically confirmed IBD (UC or CD) with active disease or clinical remission, the patient can understand and answer questions, and the patient can sign the informed consent form.

A total of 244 IBD patients identified from the Genome Database of the Latvian Population were included in the study after obtaining informed consent and the completion of health and heredity questionnaires. The study was performed in accordance with the Declaration of Helsinki and was approved by the Central Medical Ethics committee of Latvia (protocol no. 22.03.07/A7, no. 3/18-02-21).

For the first study, the TPMT genotype was detected for all 244 IBD patients. In the second study, the TPMT phenotype was investigated for 20 patients. In the third study, 50 patients were analysed for malnutrition risk factors and treatment with a control group of 50 patients.

2.2. Patient data and information acquisition

Patients' data were anonymised by assigning individual patient codes. In the first and second studies, the protocol included age, gender, weight, and height data from the patient's medical card; as well as the duration of IBD, the history of the anamnesis, such as concomitant illness, surgical operations, smoking and drug use, medication intolerance and allergies, endoscopic examinations (colonoscopy), and interpretation of the biopsy result; blood laboratory and immunological analyses (full blood count), C-reactive protein (CRP), serum amyloid A (SAA), alkaline phosphatase (SF), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyltransferase (GGT), total protein (OBV), albumin, lipase, alpha-amylase, ferritin, iron, creatinine, and faecal examination for calprotectin.

The following data were collected for each patient based on responses to the health and heredity questionnaires: demographics, gender, age, nationality, and the region of Latvia where the patient was born (Vidzeme, Kurzeme, Latgale, or Zemgale). The questionnaires assessed the patient's medical history, lifestyle, and other important factors, such as smoking status, physical activity,

possible risk factors of anamnesis, allergic reactions, medication intolerances, regular medications, and comorbidities.

In the third study, fifty hospitalised patients were screened using both NRS2002 and MUST scores (Kondrup, 2003). The European Society for Clinical Nutrition and Metabolism (ESPEN) practical guideline: Clinical Nutrition in inflammatory bowel disease, 2019, emphasises that for adult IBD patients, the risk of malnutrition can be assessed using validated screening tools, including both NRS2002 and MUST. Both tools were developed to assess the nutritional status of patients and to predict possible outcomes associated with nutritional status. MUST assessed three factors: BMI, weight loss in the past 3–6 months, and severity of illness and NRS2002 included the same questions, but additionally evaluated nutritional intake over the week prior to assessment and had an additional point for elderly patients (i.e. aged >75 years). NRS2002 also stratified diseased patients according to the severity of the disease (Bischoff et al., 2020; Kondrup, 2003). Patients were screened twice if their scores for the first assessment indicated a nutritional risk. None of the patients had any autoimmune diseases or surgical interventions in the anamnesis. IBD patients were divided into two groups: low clinical activity indexes (CDAI <150 for CD and Mayo <4 for UC) and high clinical activity indexes (CDAI >150 for CD and Mayo >4 for UC) and were further divided into smaller groups for UC and CD separately (Figure 8).

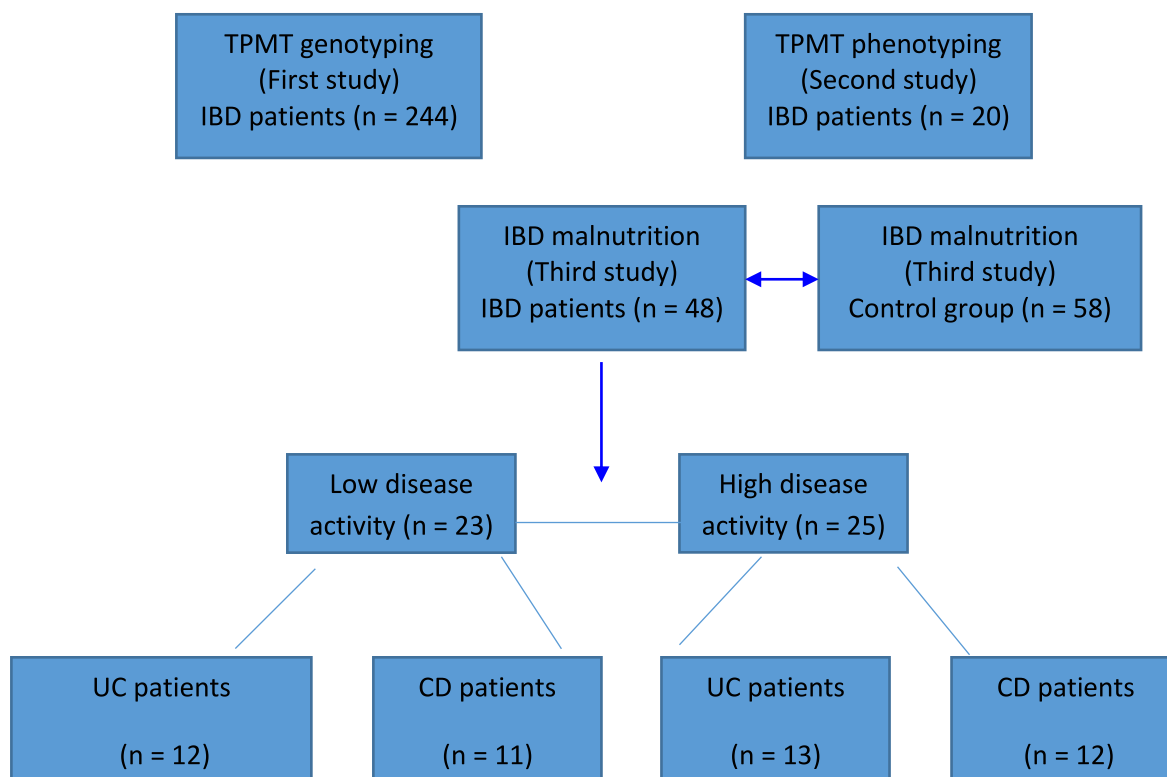


Figure 8. Diagram of study flow.

2.3. Blood samples preparation

In this study, blood samples were taken by certified medical personnel in accordance with the requirements for biological material collection and sterility. In this process, only a single system for removing blood (needles, needle holders, vacuum cleaners, etc.) was used. All patients' blood samples were collected at the Pauls Stradiņš Clinical University Hospital in the Gastroenterology, Hepatology and Nutrition Center procedure room or in the Personal Medical Laboratory according to the same methodology. From each IBD patient, we collected 20 mL of blood from the elbow vein to a vacutainer-type, ethylenediaminetetraacetic acid-containing tube and 7 mL of blood to a clot-activator tube. Serum, plasma, and white blood cells were separated within 2 days of blood collection and stored in several aliquots with transport boxes. DNA was extracted using the phenol-chloroform extraction method. The samples/aliquots of plasma, serum, white blood cells and DNA were stored and frozen at −80°C to avoid repeated cycles of re-freezing and defrosting. All tubes with blood samples were placed on a special stand, preventing them from tipping over during storage and transport.

2.4. TPMT genotype detection

TPMT genotypes were determined by quantitative real-time polymerase chain reaction (qPCR) using TaqMan Fluorescent Probes (TaqMan Drug Metabolism Genotyping Assays) for detection of the rs1800462, rs1800460, and rs1142345 SNPs. The three common non-functional TPMT alleles (TPMT * 2, * 3B, and * 3C) were determined. The PCR reactions amplified probes binding to DNA copies at sites of the TPMT gene that might contain polymorphisms and emitted fluorescent signals. All polymorphisms were analysed using a StepOneTM Software version 2.3 Real-Time PCR System. TPMT * 2, *3 A, * 3B, and * 3C allelic variants detected by qPCR were confirmed via the PCR-restriction fragment length polymorphism technique and allele-specific PCR. The results of qPCR and the alternative PCR assays were consistent. The DNA fragments were separated and analysed in 2.5% agarose gel and visualised by staining with ethidium bromide.

2.5 TPMT phenotype detection

The samples were centrifuged 30 min after collection for 15 min at 1000 rpm at 4 °C. TPMT expression was determined by enzyme-linked immunosorbent assay (ELISA) using the MyBioSource reagent kit Human TPMT ELISA Kit (catalogue number MBS938845). The kit included: antibody biotin (100-fold concentrate; 120 µL), biotin diluent (15 mL), avidin HRP or horseradish peroxidase (100-fold concentrate; 120 µL), avidin HRP diluent (15 mL), standard solution, sample diluent (50

mL), wash buffer (25-fold concentrate; 20 mL); TMB or tetramethylbenzidine substrate (10 mL); suspension solution (10 mL); adhesive strips (4 pieces); 96-field plate. A TECAN Infinite 200 PRO spectrometer was used to measure light absorption.

2.6. Bioelectrical impedance analysis method

BIA was performed for 48 patients on admission day. BIA was not carried out for two patients, because they were bedridden due to severe exacerbation of their underlying illness. The GENIUS 2002 (Jawon Medical, Korea) was used to assess BIA, and measurements were performed at least 3 h after eating to reduce small errors in impedance determination (Kyle, 2004). The instrument required the patient to be in the standing position and used eight touch electrodes with frequency ranges of 5, 50, and 250 kHz to take measurements. Electrical currents can penetrate tissues in various frequencies based on their different electrical features and the hydration or nutritional status of the patient; multifrequency BIA increases the accuracy of measurements (Kyle, 2004). The whole body was measured by subdividing it into various segments. Furthermore, weight (kg), BMI, FFM (kg), soft lean mass (SLM) (kg), metabolic body fat (MBF) (kg), total body water (TBW) (kg), percent body fat (PBF) (%), basal metabolic rate (kcal), total energy expenditure (kcal), and visceral fat (%) were assessed.

2.7. Control group

For the third study, 58 individuals from the general population were enrolled as the control group. BIA was performed if the patient met the inclusion criteria: age ≥ 18 years old and age-matched to the patient group, with no food intake ≥ 3 h prior to testing, and with no nutritional risks identified using both screening tools (NRS2002 and MUST). These criteria ensured that the control group did not harbour any active disease that would interfere with the study results. After exclusion, the control group comprised 48 individuals; the selection ensured appropriate age- and sex-matched (i.e. with equal male to female ratio) controls for the study group.

2.8. Statistical analysis

For the statistical analysis, we used SPSS version 23.0 (IMDB Statistical Package for the Social Sciences 23.0). Statistical significance was set at p-values ≤ 0.05. Data are indicated as median

(interquartile range (IQR) 25–75th percentile) or mean ± SD for normally distributed data sets. The independent sample Kruskal–Wallis test was used to identify statistically significant differences between groups of independent variables for non-parametric datasets while one-way ANOVA was used for data with normal distribution. After non-paired analysis, the Mann–Whitney U test was performed to evaluate variance between pair-matched groups. For related samples, the Wilcoxon test was used to compare two series of scores in the same group.

Continuous variables were presented as the median and interquartile range (Q1−Q3) and were compared using the Mann–Whitney test. The categorical variables were expressed as the frequency and percentage and were compared using Pearson's chi-squared test with Fisher's exact test or Cramer's V effect size as appropriate. Odds ratios (ORs) were presented with 95% confidence intervals (CI).

3. RESULTS

3.1. TPMT genotyping (First study)

3.1.1. Study participants

Our study population comprised 244 adult IBD patients, with an almost equal gender ratio (51% women and 49% men). The mean age of the participants was 43 ± 16 years. Among these patients, 78%, with a median age of 41 years (Q1−Q3 = 29.8−54.3), had UC, and 22%, with a median age of 43 years (Q1−Q3 = 30.8−55.0), had CD (p = 0.57) (Figure 9). Women comprised 47% and 63% of the UC and CD groups, respectively (Table 4).

Table 4. Characteristics of the study group.

Diagnosis	UC	*N*	190
		%	77.9%
	CD	*N*	54
		%	22.1%
Sex	Female	*N*	124
		%	50.8%
	Male	*N*	120
		%	49.2%
Age	Median		41
	Min		17
	Max		82
Age group	Age <50	*N*	187
		%	76.6%
	Age >50	*N*	57
		%	23%
Brothers/Sisters		*N*	178
		%	73%
Twin brothers/Twin sisters		*N*	4
		%	0.02%

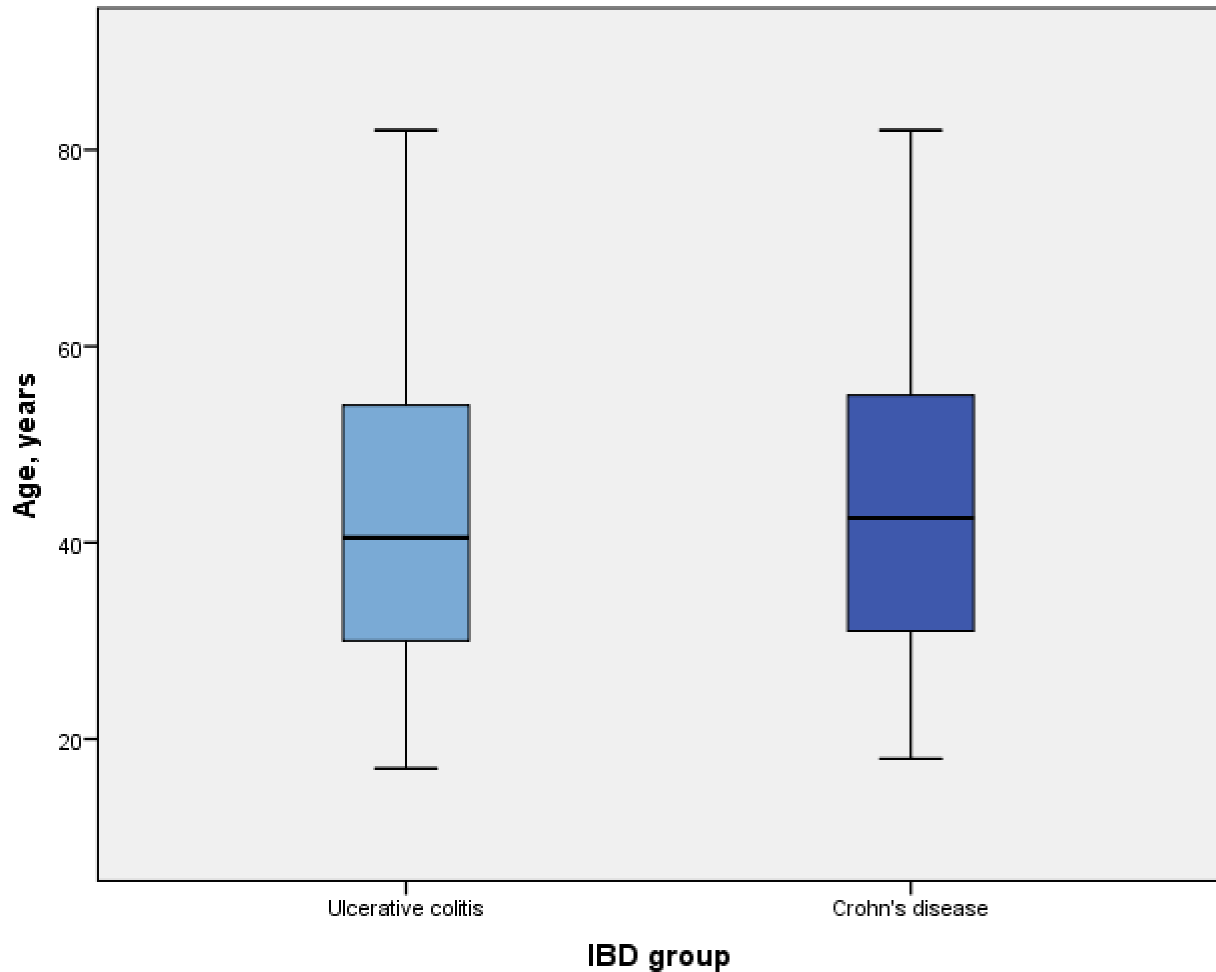


Figure 9. IBD median age.

3.1.2. TPMT genotyping

TPMT alleles were identified in all 244 patients, with 93.9% carrying a wild-type homozygous TPMT *1/*1 genotype and 6.1% were heterozygous and harboured polymorphisms (4.9% of whom had UC) (Table 5). However, we found that TPMT polymorphisms were not consistently associated with IBD (OR: 1.15, 95% CI: 0.31−4.28, p = 0.99). The most frequent polymorphisms (5.3%) were TPMT *1/*3A genotype with TPMT *3B and TPMT *3C alleles (Table 5). Only two patients had TPMT *1/*3C and TPMT *1/*2 genotypes independently. No patients carried the TPMT *3B polymorphism and no patient was found to be homozygous for any mutation (Figure 10).

Table 5. Distribution of major TPMT alleles.

	Frequency of alleles (%)	Patients, *N*
TPMT *1/*1	93.9	229
TPMT *1/*3A	5.3	13
TPMT *1/*3C	0.4	1
TPMT *1/*2	0.4	1
Total heterozygous genotypes	6.1	15

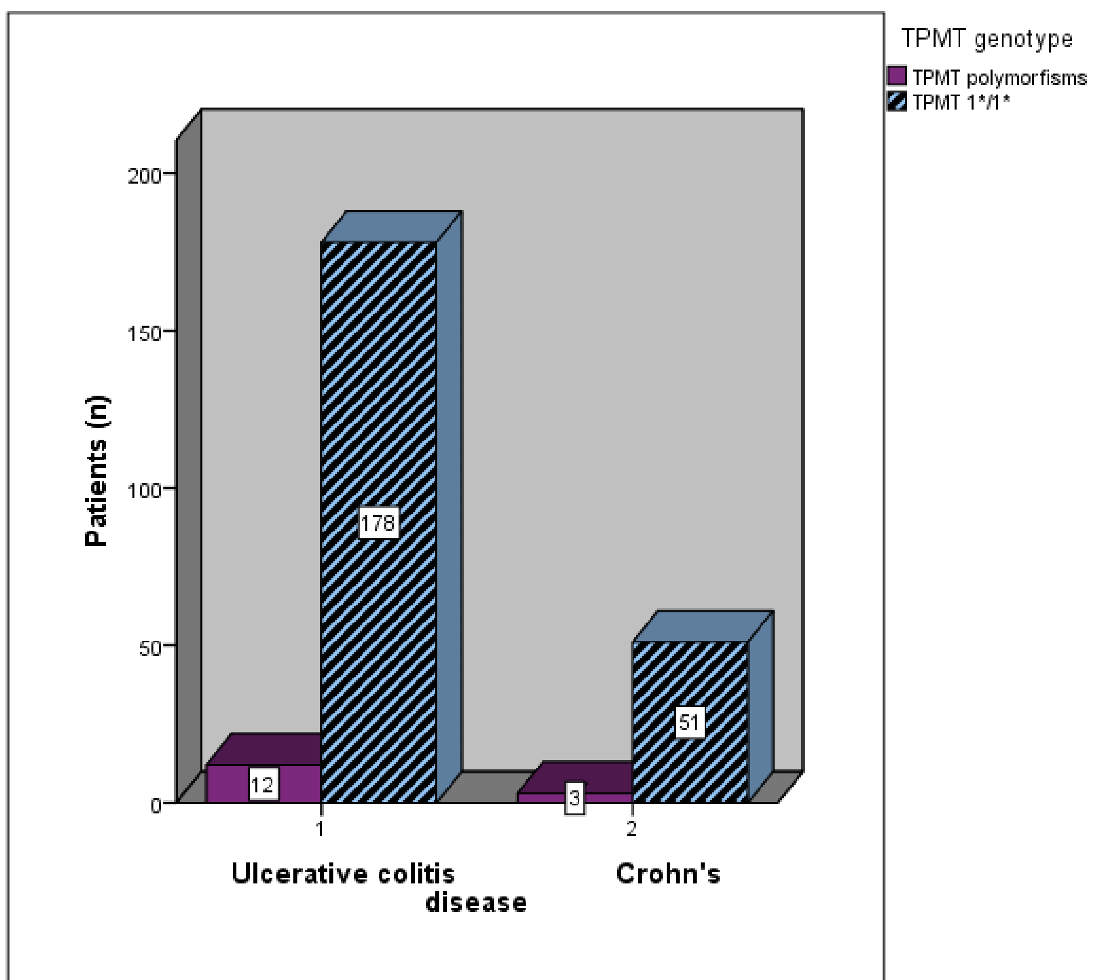


Figure 10. TPMT genotype in IBD groups.

3.1.3. Association of TPMT polymorphisms with different clinical factors

We examined whether different clinical factors were associated with TPMT polymorphisms (Table 6). We observed no significant association between gender and TPMT polymorphisms (p = 0.21). Most patients with TPMT polymorphisms were Caucasian and were born in Latvia, outside the capital Riga. A moderate association was found for patients born in the Vidzeme region (Cramer's V = 0.2). Fisher's exact test showed a nominal statistical association between TPMT polymorphisms and possible risk factors of anamnesis (working with chemicals, dust, aerosols, and lacquers, and working in chemical factories) (p = 0.04).

Table 6. Patients' diagnosis and region of residence in different TPMT allele subgroups.

		TPMT genotype				
		Heterozygous		Wild-type		
		N	% of total	*N*	% of total	*p value*
IBD group	UC	12	4.9%	178	73.0%	
	CD	3	1.2%	51	20.9%	0.99
Region of Latvia	Kurzeme	2	0.9%	32	15.2%	
	Vidzeme	5	2.4%	130	61.6%	
	Latgale	1	0.5%	23	10.9%	
	Zemgale	4	1.9%	14	6.6%	0.02
City	Riga	5	2.4%	81	38.4%	
	Outside of Riga	7	3.3%	118	55.9%	0.99
Other countries	Russia	1	3.0%	13	39.4%	
	Belarus	1	3.0%	7	21.2%	
	Ukraine	0	0.0%	4	12.1%	
	Estonia	1	3.0%	0	0.0%	
	Lithuanian	0	0.0%	2	6.1%	
	Other	0	0.0%	4	12.1%	0.05
Azathioprine	Receive	0	0.0%	22	9.0%	
	Do not receive	15	6.1%	207	84.8%	0.38

Among all included patients, 80% regularly took IBD medication and 18% had experienced allergic reactions to antibiotics, analgesics, and other drugs. Although no statistical association was found between TPMT polymorphisms and drug allergy (p = 0.78), 0.8% of patients with positive

TPMT polymorphisms (TPMT *1/*3A genotype) had previously used AZA and had experienced adverse drug reactions, such as myelosuppression and gastrointestinal intolerance.

Myelosuppression was characterised as decreased white blood cells (neutropenia <1,500 neutrophils/µL of blood) and gastrointestinal intolerance was characterised as vomiting, nausea and stomach cramps. Furthermore, we found that 15% of all the patients were smokers with a median smoking duration of 14 years (Q1–Q3 = 10.0–30.0) and 28% of the patients were previous smokers with a median smoking duration of 10 years (Q1–Q3 = 4.0–20.0). However, Fisher's exact test did not reveal any statistical associations between TPMT polymorphisms and smoking status (OR: 1.19, 95% CI: 1.12–1.26, p = 0.14).

3.2. Method of detection of thiopurine methyltransferase polymorphisms

(Latvia patent registry Nr. 15508 B, 2021)

3.2.1. Optimising the method of TPMT genotype detection

As described in Section 1.3. before the literature data, the TPMT gene has many polymorphisms that affect thiopurine metabolism. Our polymorphism detection method considered three well-described mutations of the highest incidence compared to other TPMT mutations that also impact thiopurine drug therapy. Discussions about the economic benefits of conducting a TPMT genotype test on every patient that starts thiopurine drug treatment is still ongoing. TPMT genotype testing recently became available in Latvia; however, it remains expensive and is not covered by health authorities. We aimed to develop a low-cost and reliable TPMT testing method by combining commercially available reagents from different manufacturers. We developed a qPCR method and provided instructions for reaction mix preparation and the optimised qPCR cycling program.

The typical qPCR mixture includes purified patient DNA, reaction buffer containing Mg2+ ions, a polymerase that performs copying of the target gene, primers that anneal to the target gene, and a probe that anneals within an amplified DNA region and emits fluorescent light that serves as a signal for DNA amplification. The target gene is a gene of interest that in some cases contains mutation/deletion/polymorphism, but in most cases does not contain alterations and is called a wild-type gene.

For studies of novel mutations, there are computer programmes available that help scientists design unique primers and probes that can be synthesised and ordered from manufacturers. Normally, several sets of these oligonucleotides are ordered and used in reactions where timing and temperature

vary. The concentration of components is adjusted experimentally, and different reaction buffers can be used to optimise the reaction. The qPCR was considered as optimised when:

1. A strong fluorescent signal was emitted for positive samples.

2. Very little signal was detected for negative samples, indicating the clear difference between the test results of samples containing variation and wild-type genes.

3. Testing costs were reduced.

4. The protocol was handy and easy work to minimise the possibility of errors.

We bought commercially available PCR reagents for the detection of TPMT polymorphisms. American company Applied Biosystems (now part of Thermo Fisher Scientific) holds a solid reputation in quality research and offer millions of SNP assays, including assays for the detection of TPMT polymorphisms. The assays from this company met our needs in several ways:

1. TaqMan SNP Genotyping Assays have a unified qPCR set-up protocol that allows running PCR reactions simultaneously to detect different TPMT polymorphisms,

2. TaqMan assays contain probes and primers in one mixture. There are only three reaction components for PCR: purified genomic DNA (1–20 ng), assay solution, and TaqMan Genotyping Master Mix (or another compatible master mix),

3. Reasonable price and number of reagents to test the hundreds of patients in our study.

TaqMan assays are marked for research purposes only; however, they are used in clinical settings. The price is much lower for reagents marked for research purposes compared to in vitro diagnostic (IVD) reagents, but it does not necessarily mean that they perform worse. The use of IVD reagents in clinical laboratory settings requires strict adherence to manufacturers' instructions starting from sample collection, recommended DNA isolation kits and equipment, and the use of a particular PCR analyser. Moreover, the manufacturers of IVD reagents restrict their liability by stating that results might be false positive or false negative. We chose to optimise the reaction protocol and verify the method ourselves.

3.2.2. Characterisation of probes and primers used in our protocol

TaqMan™ SNP Genotyping Assays employ TaqMan 5′ nuclease chemistry for amplifying and detecting specific SNP alleles, multi-nucleotide polymorphisms, and insertion/deletions.

We used three TaqMan SNP Genotyping Assays (Table 7). TPMT *3A is 3B with 3C in one patient. All three SNP are in chromosome 6. All TaqMan Assays have a FAM or VIC reporter dye at the 5′ end of each probe and a nonfluorescent quencher at the 3′ end of each probe. The reporter 1 (allele1) dye was always VIC and the reporter 2 (allele2) dye was always FAM.

Table 7. Information from TaqMan Assays Safety Data Sheet (SDS).

	SNP	Mutation type	Probe
TPMT *2 (G238C)	rs1800462	Mis-sense	C_12091552_30
Context Sequence and the targeted SNP in square brackets	CCAACTACACTGTGTCCCCGGTCTG[C/G]AAACCTGCATAAAATCATACATTTA		
TPMT *3B (G460A)	rs1800460	Mis-sense	C_30634116_20
Context Sequence and the targeted SNP in square brackets	TCACCTGGATTGATGGCAACTAATG[T/C]TCCTCTATCCCAAATCATGTCAAAT		
TPMT *3C (A719G)	rs1142345	Intergenic/unknown	C-19567_20
Context Sequence and targeted SNP in square brackets	TCTCATTTACTTTTCTGTAAGTAGA[C/T]ATAACTTTTCAAAAAGACAGTCAAT		

Cycling conditions and reaction setup for TaqMan Assays are described in Table 8. The genotyping experiments were performed according to the recommendations of Applied Biosystems described in manual TaqMan® SNP Genotyping Assays (Revision A.O) (TaqMan® SNP Genotyping Assays User Guide - Pub. no. MAN0009593 - Rev. A.0).

Table 8. Recommended thermal cycling settings we used in our study.

Step	Temperature (°C)	Duration	Cycles
Polymerase activation	95	10 min	HOLD
Denaturation	95	15 s	40
Annealing/Extension	60	1 min	

Table 9. Recommended preparation of reaction mix we used in our study at the beginning.

Component	Volume for 96-well plate (µL)
2x TaqMan Master Mix**	12.5
20x Assay	1.25
DNA in nuclease-free water*	11.25
Total volume per well	25

* Recommended amount of DNA is 1–20 ng per reaction. We added up to 100 ng DNA in the reaction mix without compromising allelic discrimination results.

**We used 2x TaqMan Universal PCR Master Mix

To reduce testing costs, we started with sets of experiments with lower reaction volumes than specified by the manufacturers and lower concentrations of TaqMan 20x Assays (probes and primers) compared to the recommended values. Specifically:

1. Reaction mix volume was reduced to approximately 10 µL of total reaction volume.
2. 20x Assay were diluted 1:10 with nuclease-free water.
3. TaqMan Master Mix was substituted with Solis BioDyne HotFirePol Master Mix.

Each modification was introduced step-by-step by testing the same samples in different experiments or by parallel sample testing in reactions of varied volumes and probe concentrations on one reaction plate.

While cost-reduction was a goal, we ensured that the orientation of VIC® -dye and/or FAM™ -dye clusters in allelic discrimination plots remained distinct to distinguish wild-type alleles from polymorphisms (Figure 11).

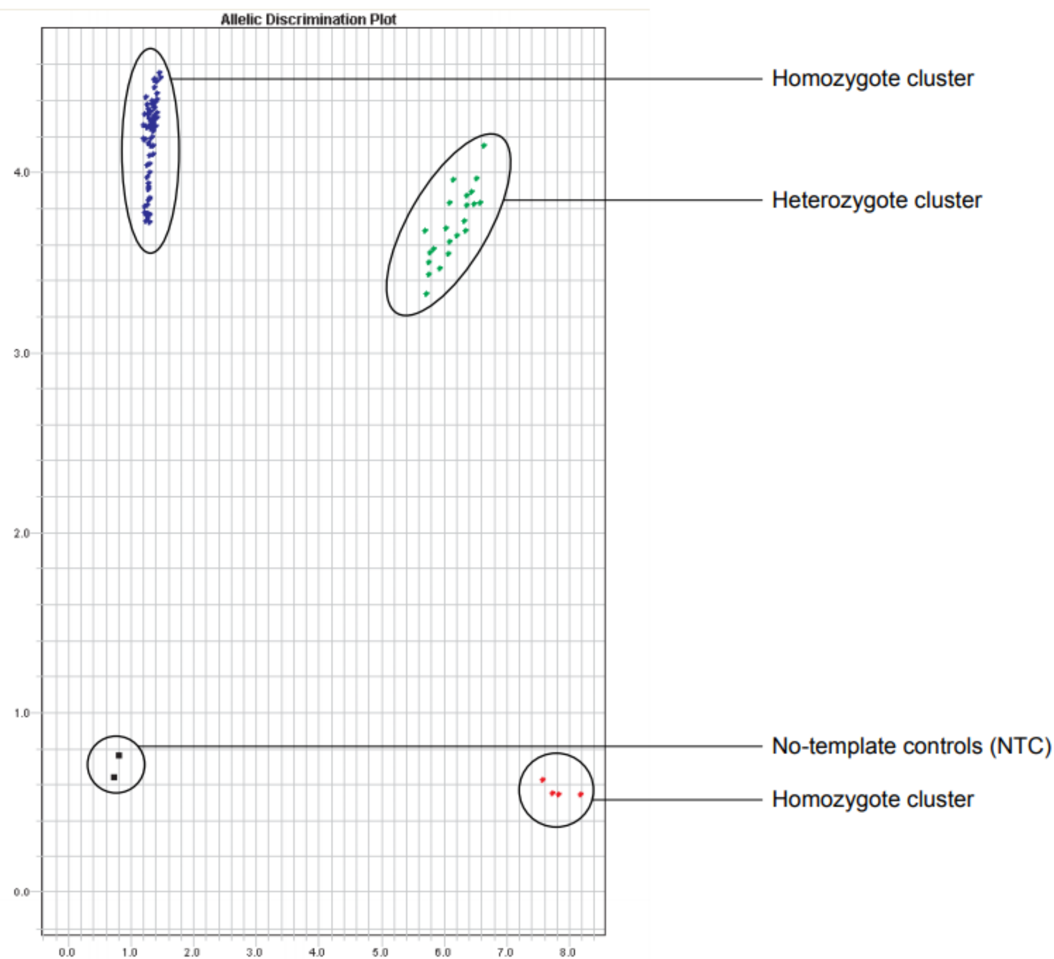


Figure 11. Typical allelic discrimination plot. Ideally, the points in each cluster are grouped closely together and each cluster is located well away from the other clusters. (Adapted from TaqMan® SNP Genotyping Assays User Guide - Pub. no. MAN0009593 - Rev. A.0)

Allelic discrimination experiments were performed on an Applied Biosystems StepOnePlus instrument. qPCR reactions were set up in 0.1-mL tubes (consumables) designed for 96-well heating plate thermal cyclers.

3.2.3. The patented method of TPMT testing

The optimised TPMT method was finalised with patent Nr. 15508 B1 (Method of detection of thiopurine methyltransferase polymorphisms) issued by the Patent Office of the Republic of Latvia on February 20, 2021.

The patent describes the preparation of two reaction mixes and qPCR cycling conditions for testing TPMT *2, *3B, and *3C polymorphisms (Tables 10 and 11). Patented cycling conditions are described in Table 12.

Table 10. First patented reaction mixes.

Component	Volume
Master Mix (2x)*	5 µL
TaqMan Assay	0.125 µL
H_2O	4 µL
DNA	1 µL (3–100 ng)
Total	10.125 µL

*2x TaqMan Universal PCR Master Mix

Table 11. Second patented reaction mixes.

Component	Volume
Master Mix (5x)*	2 µL
TaqMan Assay	0.125 µL
H_2O	7 µL
DNA	1 µL (3–100 ng)
Total	10.125 µL

* Solis BioDyne 5x HOT FIREPol® Probe Universal qPCR Mix

Table 12. Patented cycling conditions specified for both reaction mixes

<table>
<tr><th>Step</th><th>Temperature (°C)</th><th>Duration</th><th></th></tr>
<tr><td>Polymerase activation</td><td>95</td><td>10 min</td><td></td></tr>
<tr><td>Denaturation</td><td>92</td><td>15 s</td><td rowspan="2">40 cycles</td></tr>
<tr><td>Annealing/Extension</td><td>60</td><td>60 s</td></tr>
</table>

3.2.3.1. Extraction and purification of DNA

The IBD patient samples (244 samples) used in our study were obtained from the Genome Database of Latvian population (LGDB), a national biobank of Latvian Biomedical Research and Study Centre. LGDB uses the phenol-chloroform DNA extraction method to isolate genomic DNA from blood.

Most blood samples came from Pauls Stradiņš Clinical University Hospital. DNA from the samples were extracted using Automated nucleic acid extraction system Maxwell 16 (Promega) that purifies samples using paramagnetic particles. Paramagnetic beads provide a mobile solid phase that optimises capture, washing and eluting the target material.

DNA was isolated using Maxwell® 16 Blood DNA Purification Kit (Promega) for whole blood or buffy coat samples. We isolated DNA from 400 µL of blood samples collected in EDTA. Fresh blood samples were stored at 4 °C and processed within 7 days of the collection according to the Maxwell(R) 16 DNA Purification Kits Technical Manual. The automated system was user-friendly and isolated DNA in about 50 min. The concentration of DNA was determined using a Colibri spectrophotometer.

3.2.3.2. Evaluation of the TPMT testing qPCR method

Evaluation is a generic term used to describe the measurement of the performance capabilities of a system/test method. This is a process that compares different systems/test methods designed to perform the same or similar functions. There are no strict common standards to evaluate testing methods, and laboratories differ in ways of ensuring that their testing method is robust and precise. Normally, this is based on a comparison of test results with other methods, reference samples, clinical data, or results from other laboratories. Our laboratory participated in external quality assessment (EQA) and compared all qPCR positive testing results and several wild-type samples to alternative PCR methods.

3.2.3.3. External Quality Assessment

Verification under conditions may be demonstrated by participation in a performance testing program, provided that the tested material is representative of the method (C6795744 (demarcheiso17025.com))

In 2018, our laboratory participated in EQA of the reference laboratory Instand in Germany. The EQA was designed to verify the capacity of the laboratory to detect TPMT *2, TPMT *3B, and

TPMT *3C polymorphisms in clinical settings. We ordered three samples (Set 06 from group 775) and correctly identified two wild-type samples and one sample carrying 3B and 3C polymorphisms.

3.2.4. Comparison with alternative PCR methods

We checked and approved the TPMT *2, *3A, *3B, *3C polymorphisms found with allele-specific PCR and restriction fragment length polymorphism (RFLP) methods with minor modifications. The modifications were applicable to the polymerase type, polymerase buffers, and restriction enzyme (restrictases) buffers used in our study.

An allele-specific PCR was employed to analyse the TPMT *2 polymorphism, and a PCR-RFLP method for the detection of TPMT *3B and *3C variations.

Nineteen patient samples previously tested with the qPCR method were checked with allele-specific PCR and RFLP methods. One sample had TPMT *2 and one had TPMT *3B polymorphism, 13 samples were positive for TPMT *3A and four were wild-type samples. There was full agreement between the qPCR and PCR/RFLP results.

PCR samples were incubated in TProfessional Gradient thermocycler (Biometra) and PCR products were visualised in 1.5% agarose gel in TBE buffer.

*3.2.4.1. Detection of TPMT *2*

Several sets of experiments were performed to optimise the cycling temperature and $MgCl_2$ concentration in the reaction mix (Table 13 and 14). Primers were ordered from Metabion International AG and the primer sequences are described in a previous report (Ameyaw et al., 1999).

Table 13. PCR cycles.

Cycle step	Temperature	Time	Cycles	Recommended settings by Solis BioDyne
	Our settings			
Initial activation	95 °C	13 min	1	12–15 min
Denaturation	95 °C	15 s	31	15–30 s
Annealing	see below	40 s		30–60 s; 50–68 °C; 26–35 cycles
Elongation	72 °C	30 s		1–4 min
Final elongation	72 °C	5 min	1	5–10 min

Table 14. Adjusting the annealing temperature and $MgCl_2$concentration in a 20-µL reaction mix.

<table>
<tr><th></th><th>Annealing t°</th><th colspan="3">Volume of $MgCl_2$ (10 mM)</th></tr>
<tr><td>1</td><td>52</td><td rowspan="6">1.2 µL</td><td rowspan="6">1.6 µL</td><td rowspan="6">2 µL</td></tr>
<tr><td>2</td><td>53.5</td></tr>
<tr><td>3</td><td>57.1</td></tr>
<tr><td>4</td><td>61.0</td></tr>
<tr><td>5</td><td>64.8</td></tr>
<tr><td>6</td><td>67.6</td></tr>
</table>

The highest yield of amplified DNA can be achieved by 0.8 mM $MgCl_2$ concentration (1.6 µL $MgCl_2$ in 20-µL reaction mix) at an annealing temperature range of 52–61 °C (Figure 12).

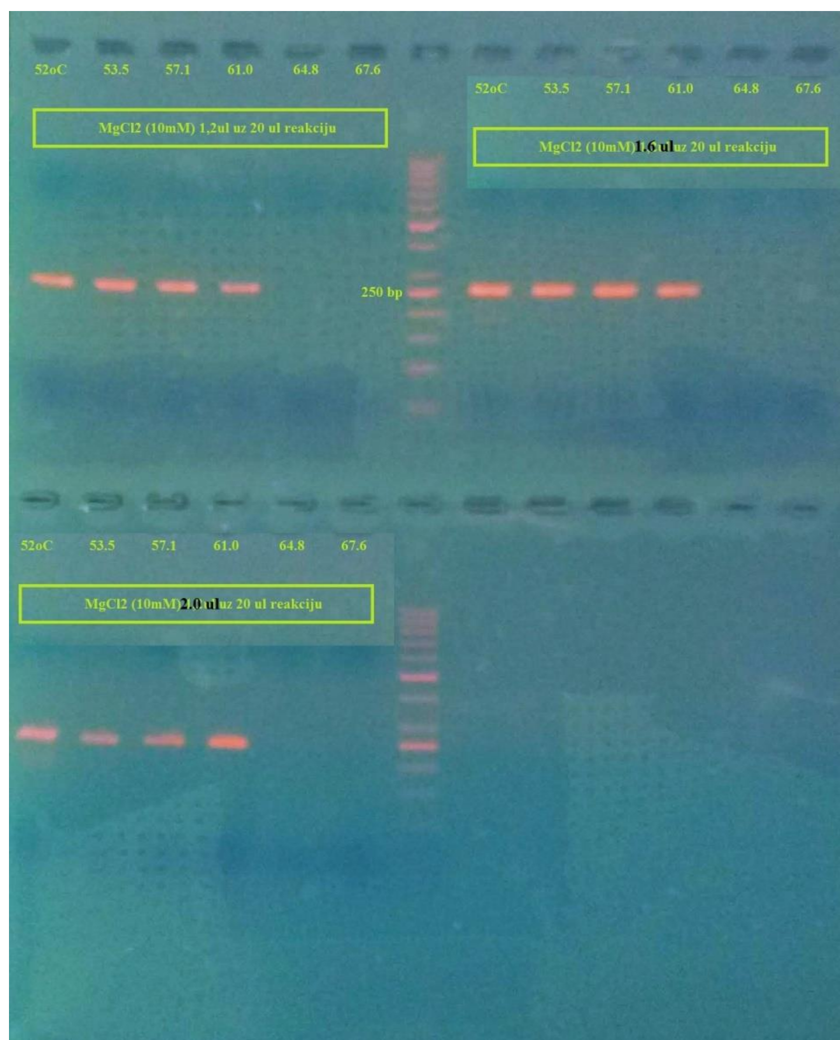


Figure 12. TPMT *2 PCR products detected in agarose gel electrophoresis. The presence of TPMT *2 polymorphism resulted in the amplification of 254-bp fragments that are visible in the agarose gel under UV light. PCR conditions such as temperature and $MgCl_2$ concentration affect the amount of PCR product and intensity of fluorescence emitted by the DNA dye (ethidium bromide).

Two forward primers, P2W (5′-GTA TGA TTT TAT GCA GGT TTG-3′) and P2M (5′-GTA TGA TTT TAT GCA GGT TTC-3′), were used with one reverse primer P2C (5′-TAA ATA GGA ACC ATC GGA CAC-3′) in wild-type-specific and mutant-specific reactions, respectively. The PCR products (254 bp) were analysed on a 1.5% agarose gel. Among six patient samples, one positive for TPMT *2 and five negative in qPCR were retested by the allele-specific PCR method. Each patient's DNA was tested using two PCR reaction mixes that differed in forward primers, P2W or P2M. The patient previously tested positive has both wild-type and mutation-specific reaction products, indicating TPMT *2 polymorphism in one allele, the rest are wild-type samples.

*3.2.4.2. Detection of TPMT *3B and TPMT *3C*

Allele-specific PCR and RFLP were performed using a series of steps briefly outlined below:

1. DNA extraction from blood and PCR to amplify the DNA fragment containing the variation of interest to the level required for RFLP analysis.
2. Digestion of PCR fragments with specific restriction endonucleases (restrictases). Restrictases cleave PCR products specifically in the site of point mutation. For example, if point mutation is present in the sample, then the restrictase cleaves the DNA product into two fragments of a particular length. These fragments can be seen as two separate lines in gel electrophoresis. In contrast, wild-type DNA will not be cleaved and will be visualised as a single line. Heterozygous samples contain one allele with polymorphism and one wild-type allele, therefore two cleaved fragments and one full-length fragment will be visible in gel electrophoresis. The scenario described can work oppositely, particularly with TPMT *3 when the wild-type PCR product is cleaved in two fragments, but SNP destroys the cleavage site, resulting in an uncleaned PCR product.
3. Digested and/or uncleaned DNS is transferred to agarose gel to separate the digestion products from each other.

The PCR for the detection of TPMT *3B was performed using forward primer 5′-AGG CAG CTA GGG AAA AAG AAA GGT G-3′ identical to nucleotides 756–780 of intron 6 and reverse primer 5′-CAA GCC TTA TAG CCT TAC ACC CAG G-3′ reverse complement of nucleotides 1425–1449 of intron 7. The cycling settings and reaction mix composition were adapted to the recommendations of the manufacturer of the polymerase used in our study (Tables 15 and 16).

Table 15. Reaction mix.

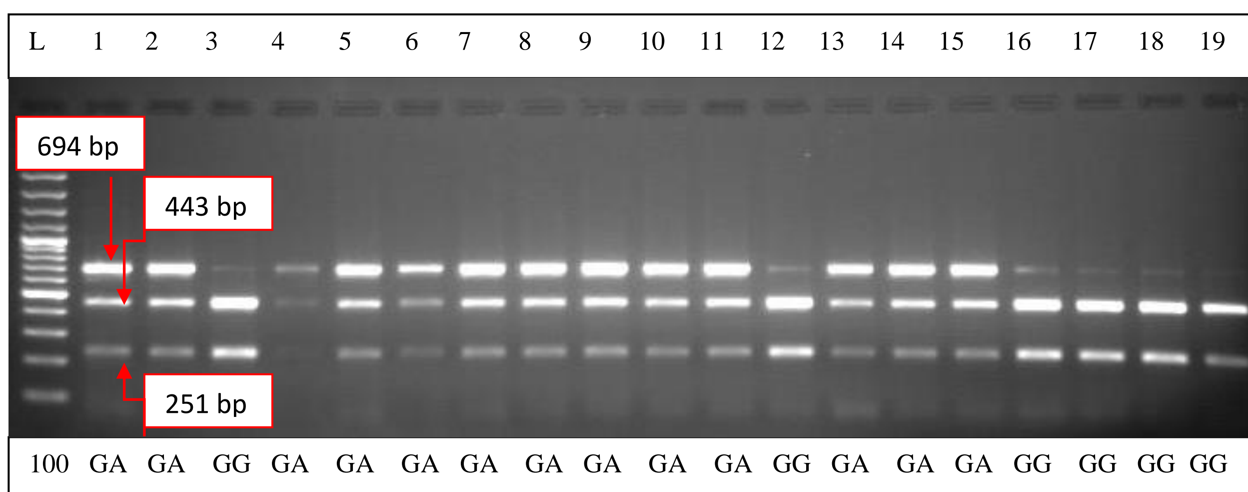
Component	Volume
Master Mix (5x)*	2 µL
TaqMan Assay	0.125 µL
H2O	7 µL
DNA	1 µL (3–100 ng)
Total	10.125 µL

* Solis BioDyne 5x HOT FIREPol® Probe Universal qPCR Mix

Table 16. Cycling conditions

Step	Temperature (°C)	Duration	
Polymerase activation	95	10 min	
Denaturation	92	15 s	40 cycles
Annealing/Extension	60	60 s	

This was followed by digestion of a 694-bp PCR product with restriction enzyme HpyF10VI (*Mwo*I) (Thermo Scientific) for 1 h at 60 °C. Digested products were separated on a 1.5% agarose gel. *Mwo*I digestion of wild-type DNA yielded fragments of 443 and 251 bp. The TPMT *3B SNP destroys the restriction site and *Mwo*I digestion yielded an uncleaned fragment of 694 bp (Figure 13).



*3A *3A N *3A *3A *3A *3C *3A *3A *3A *3A *2 *3A *3A *3A N N N N

Figure 13. TPMT *3B detection in the PCR-RFLP-based assay followed by agarose gel electrophoresis. Lane L, 100-bp DNA ladder; lanes 1–19, patient samples. Long, uncleaned PCR

product of 694 bp is characteristic of the mutant gene (lanes 1, 2, 4–11 and 13–-15); two cleavage products of 443 and 251 bp are produced by restrictase in the wild-type gene. Three bands of 694, 443 and 251 bp indicate that one allele is mutant and another is wild-type in mutant samples 1, 2, 5–11 and 13–15. Samples 3, 12 and 16–19 are wild-type, sample 12 contains TPMT *2 mutation that is irrelevant to TPMT *3B, and sample 4 shows a weaker fluorescent band.

For the TPMT *3C mutation (A719G mutation), the PCR assay was performed using 0.27 µM each of primers P719Fb (5′-GAG ACA GAG TTT CAC CAT CTT GG-3′), identical to nucleotides 401–423 in intron 9, and P719R (5′-CAG GCT TTA GCA TAA TTT TCA ATT CCT C-3′), the reverse complement of nucleotides 746–773 in exon 10 and buffer I (Invitrogen).

The PCR product was digested with restriction enzyme XmiI (*Acc*I) (Thermo Scientific) overnight at 37 °C and then separated on a 1.5% agarose gel. The TPMT *3C variation introduced an *Acc*I restriction site in the amplified fragment (373 bp), yielding fragments of 283 and 90 bp. Wild-type alleles were identified by uncleaned 373-bp fragments (Figure 14).

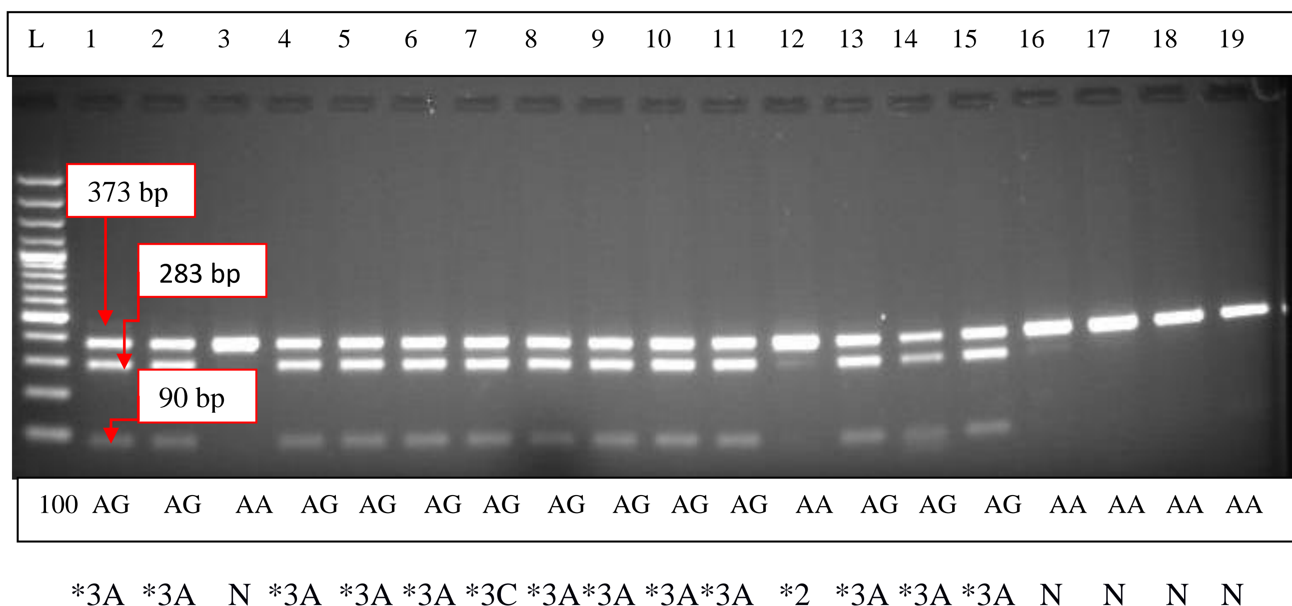


Figure 14. TPMT *3C detection by PCR-RFLP. Lane L represents 100-bp DNA ladder, lanes 1–19 represent patient samples that were previously tested positive for TPMT polymorphisms by the qPCR method (*3A, *3C or *2 in the box under the figure) and five negative samples (N). All samples have uncleaned 373-bp fragments characteristic for one or two wild-type alleles. Cleaved fragments of 283 bp and 90 bp in lanes 1, 2, 3–11 and 13–15 indicate TPMT *3C polymorphism in one allele. Therefore, all TPMT *3C mutants are heterozygous.

3.3. TPMT phenotyping (Second study)

All 20 respondents included in the study had histological diagnosis of IBD (UC in 70%, n = 14; CD in 30%, n = 6).

All patients had moderate to severe disease activity according to Mayo score in UC patients and CDAI in CD patients. 50% of respondents (n = 10) were diagnosed with IBD more than 10 years ago (Figure 15). UC was diagnosed in eight men and six women; CD was more diagnosed in five women and only one man.

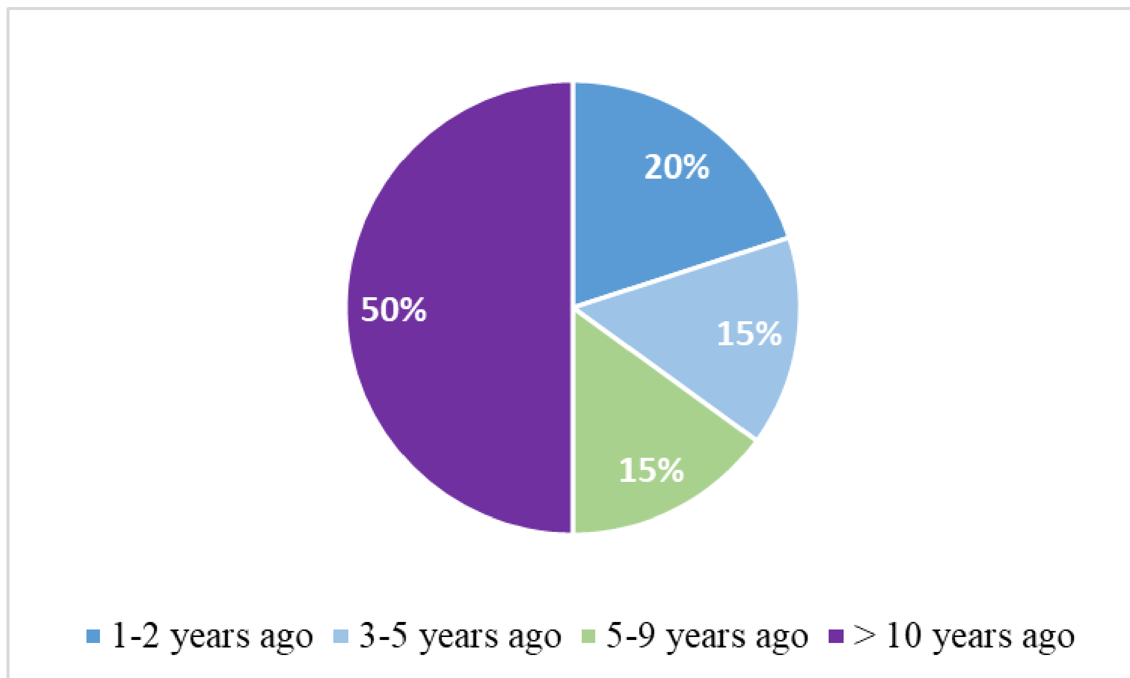


Figure 15. The distribution of respondents first diagnosed with IBD.

Summarising information on the usage of medications, 45% of respondents (n = 9) used an oral form of mesalazine and 40% (n = 8) used a combination of mesalazine in oral and suppository forms. 75% of respondents (n = 15) had never used AZA, 15% (n = 3) used it but stopped taking it due to side effects, and 10% (n = 2) were using AZA during the study (Figure 16).

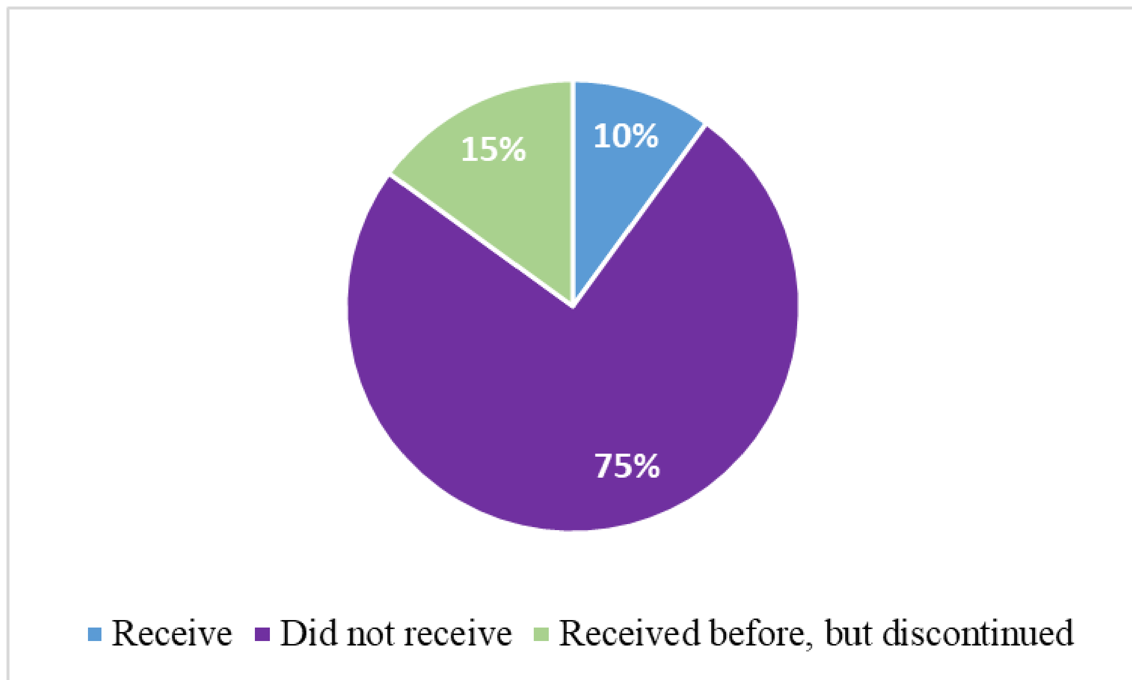


Figure 16. Division of respondents by azathioprine use.

Patients who discontinued due to adverse reactions reported side effects such as gastrointestinal symptoms and leukopenia. Patient's TPMT expression ranged from 1.4–50 U/mL. All respondents were divided by TPMT enzyme activity: 10% (n = 2) of patients had low (<5.5 U/mL) TPMT activity, 5% (n = 1) of patient had intermediate (5.6–15.5 U/mL) activity, 70% (n = 14) of patients normal (15.6–44.0 U/mL) and 15% (n = 3) of patients high (>44.0 U/mL) TPMT activity (Figure 17).

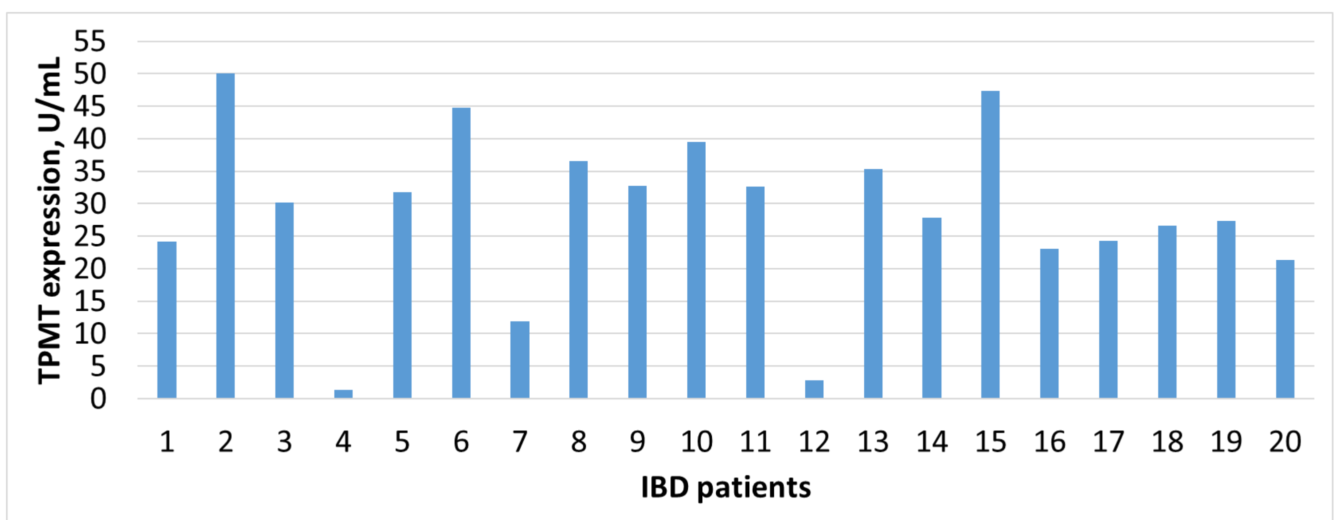


Figure 17. TPMT enzyme activity in IBD patients.

3.4. IBD malnutrition (Third study)

3.4.1. Patient study group

Among the 48 IBD patients included in the analysis, 52% (n = 25) had UC patients and 48% (n = 23) had CD. Disease activity was measured by the Mayo score for UC patients, with a median score of 4 (IQR: 1.0–6.25) and CDAI was used for CD patients, with a median result of 128 (IQR: 56.0–207.0). Of the IBD patients, 48% (n = 23) had low activity (CDAI score of <150 for CD or Mayo <4 for UC) and 52% (n = 25) had high activity (CDAI >150 for CD or Mayo >4 for UC). For IBD patients, the median age was 36.5 (IQR: 28.5–51.5). The median age for the control group was 32.0 years (IQR: 26.0–41.8), and although there was a noticeable age gap compared to the patient group, pair-matched analysis using the Kruskal–Wallis test did not show any statistically significant difference between these two groups ($p = 0.198$). Characteristics of the study group are summarised in Table 17.

Table 17. Description of the study group.

		Patients (n = 48)	Percent
Diagnosis	UC	25	52%
	CD	23	48%
Clinical activity	Asymptomatic	21	44%
	Mild	15	31%
	Moderate	11	23%
	Severe to Fulminant	1	2%
Sex	Female	19	40%
	Male	29	60%
Smoker	Yes	8	17%
	No	40	83%
Alcohol consumption	No	23	48%
	Once a week	6	13%
	Once a month	13	27%
	Less than once a month	6	13%

Table 18 shows the assessment of general laboratory variables. Patients had several micronutrient deficits, but no statistical relationship with the screening tools was identified. Nonetheless, patients who appeared to be in clinical remission and with no signs of undernutrition could still harbour micronutrient deficits.

Table 18. Comparison of laboratory tests results of low and high IBD activity groups.

	Low activity group		High activity group	
Parameter	Patients (n = 23)	Percent %	Patients (n = 25)	Percent %
CRP (>5 mg/L)	4	17.4	19	76
Albumin (<35 g/L)	6	26.1	5	20
RBC (Male <4.5 × 10^9/L; Female <4.2 × 10^9/L)	9	39.1	14	56
HTC (<40%)	14	60.9	15	60
HGB (M <130 g/L; F <120 g/L)	13	56.5	10	40
WBC count (>10 × 10^{12}/L)	4	17.4	5	20
Platelet count (>400 × 10^9/L)	5	21.7	5	20
Creatinine (<62 µmol/L)	8	34.8	7	28
Creatinine (>115 µmol/L)	1	4.3	1	4
Glucose (>6 mmol/L)	1	4.3	2	8
Ferritin (<22 ng/mL)	8	34.8	7	28

CRP, C-reactive protein; RBC, red blood cells; M, male; F, female; HTC, haematocrit; HGB, haemoglobin; WBC, white blood cells

3.4.2. Nutritional screening

Of the patients screened by NRS2002, 31% (n = 15) were revealed to be at high nutritional risk, 25% (n = 12) were at medium risk, and 44% (n = 21) were at low risk. The MUST scores were nearly inversely proportional for the high- and medium-risk groups: 40% (n = 19) of patients had a high-risk score of malnutrition, while 19% (n = 9) had a medium-risk score (Table 19).

Table 19. Comparison of risk groups according to the NRS2002 and MUST scores

Risk group	NRS2002	MUST
High-risk	31% (n = 15)	40% (n = 19)
Medium-risk	25% (n = 12)	19% (n = 9)
Low-risk	44% (n = 21)	42% (n = 20)

Despite these differences in the high- and medium-risk groups, we observed a strong positive correlation between both screening tools NRS2002 and MUST (Spearman's correlation coefficient, rho = 0.85; p <0.001) (Figure 18).

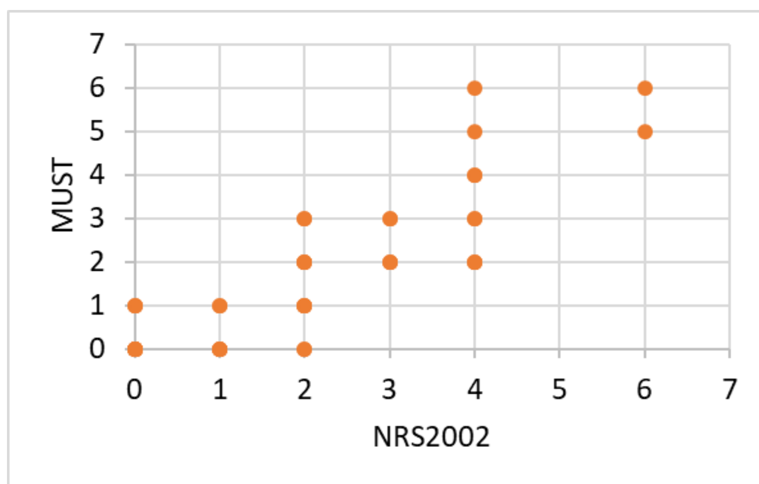


Figure 18. Spearman's correlation of MUST and NRS2002.

Previous studies have reported that disease activity significantly affects the nutritional status of patients; an increase in the activity index increases the risk of malnutrition. We observed a moderate positive correlation between the NRS2002 results and the disease activity index (Spearman's correlation coefficient, rho = 0.577; p <0.001), but only a weak positive correlation between MUST

scores and the disease activity index (Spearman's correlation coefficient, rho = 0.429; p <0.001) (Table 20).

Table 20. Spearman's correlation of activity index: NRS2002 vs MUST.

		NRS2002	MUST
Activity index	*rho*	0.577	0.429
	p-value	<0.001	<0.001
NRS2002	*rho*		0.830
	p-value		<0.001

We further evaluated whether there were significant differences between the risk of undernutrition and disease activity. A pair-matched analysis revealed a significant difference among patients in clinical remission and patients with moderate (NRS2002 p = 0.001; MUST p = 0.026, Kruskal–Wallis test) or severe disease activity (NRS2002 p = 0.023; MUST p = 0.038, Kruskal–Wallis test). In Figure 19, extreme values are shown, which indicate that patients were in clinical remission, but were still at risk of undernutrition. This reveals the importance of regular screening even if the patient's disease activity is not high. Differences in scores might be due to gradual weight loss as MUST evaluates weight changes over a 3–6-month period.

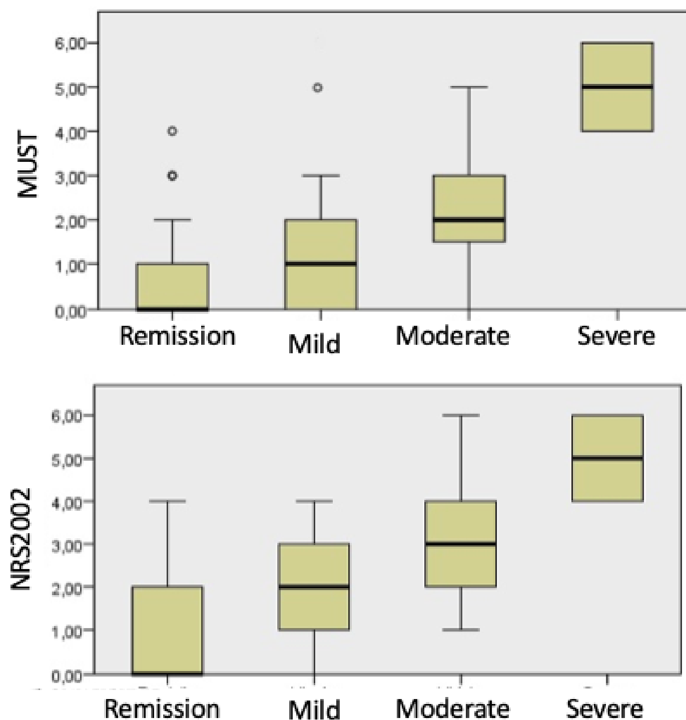


Figure 19. Screening tool results according to disease activity. *Kruskal–Wallis* test results are displayed in a box chart.

Patients at risk for malnutrition were evaluated twice using both screening tools (NRS2002 and MUST) to estimate the reduction in undernutrition risk, after receiving clinical feeding. Statistical analysis showed a reduction in malnutrition screening scores under clinical feeding (related sample Wilcoxon test, p = 0.020). However, we should consider that patients also received treatment to reduce their disease activity, which would reduce the points scored using the screening tools.

Clinical nutrition was administered to 18 patients; 17 patients were prescribed enteral oral feeding and one patient received central venous feeding. Additionally, four oral feedings patients received parenteral peripheral feeding.

The NRS2002 was used to screen 17 patients in the high-risk group; 88% (n = 15) received clinical feeding and 12% (n = 2) did not. A slightly lower percentage of patients (86% (n = 18)) received feeding of the 21 patients who were in the high-risk group based on the MUST score. Additional information on the nutritional status of the groups is presented in Table 21.

Table 21. Additional nutrition

Nutritional supplement	Contains one unit	Patients received
Nutridrink	2.4 kcal/mL, 125 mL (bottle); Fat: 11.6 g OGH: 37.1 g Protein: 12 g	- 57% (n = 8) 4 bottles/day - 28% (n = 4) 3 bottles/day - 14.2% (n = 2) 2 bottles/day
Cubitan	1.25 kcal/mL, 200 mL (bottle) Fat: 7.0 g OGH: 29 g Protein: 17.6 g	- (n = 1) 3 bottles/day - (n = 2) 2 bottles/day
Protifar	8 kcal, 1 spoon Fat 1.6 g OGH, <1.6 g	- 50% (n = 9) 3 spoons/ day
Kabiven	1448 mL, 1000 kcal, Amino acids: 456 mL, Dextrose 788 mL 13% Lipids 204 mL	- 22.2% (n = 4)

3.4.3. Bioelectrical impedance analysis

Figure 20 reveals that most patients had normal BMI values, although imbalanced body compositions with changes in percent body fat (PBF) and SLM were observed in a large proportion of patients. This indicates that even if patients have BMI within normal values, there is still a high chance that they have imbalances in body composition. Therefore, it is important that the lean mass and PBF of patients are evaluated.

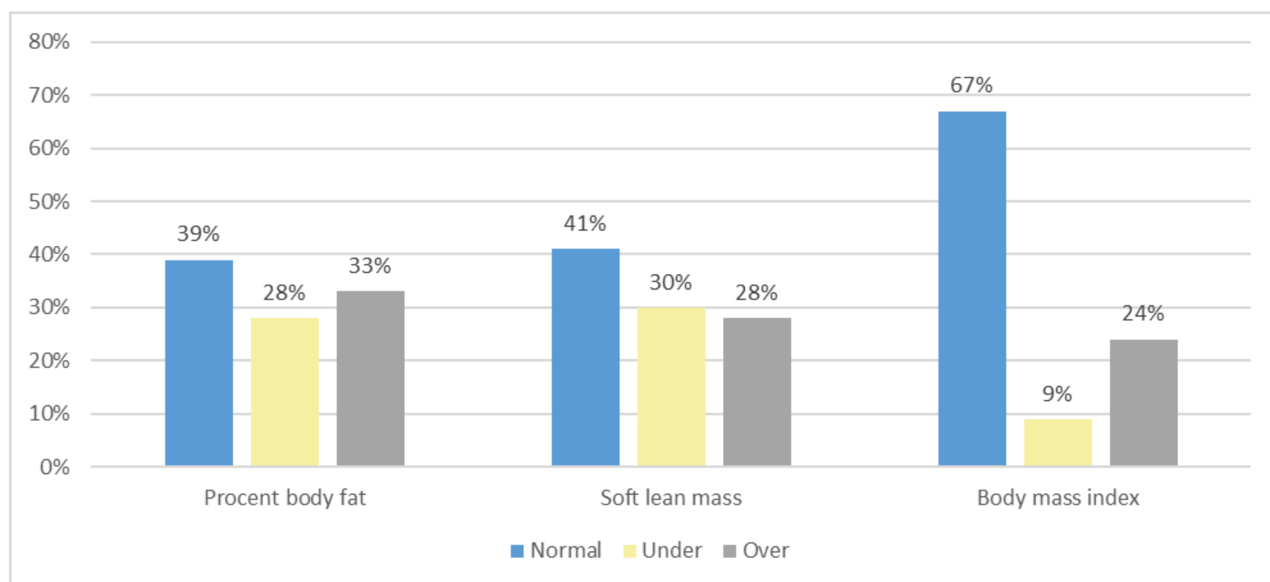


Figure 20. Differences in BIA among the patient group

Figure 21 demonstrates the changes in muscle mass from normal values. Most CD patients presented a reduction in muscle mass, which is in contrast with the greater part of UC patients that retained their normal muscle mass or presented an increase in muscle mass. While CD patients with high, low, and medium disease activity, presented decreases in muscle mass, patients were more prone to reduction in muscle mass when disease activity is high. Only a few individuals of the control group (8%; n = 4) had muscle mass under the normal values.

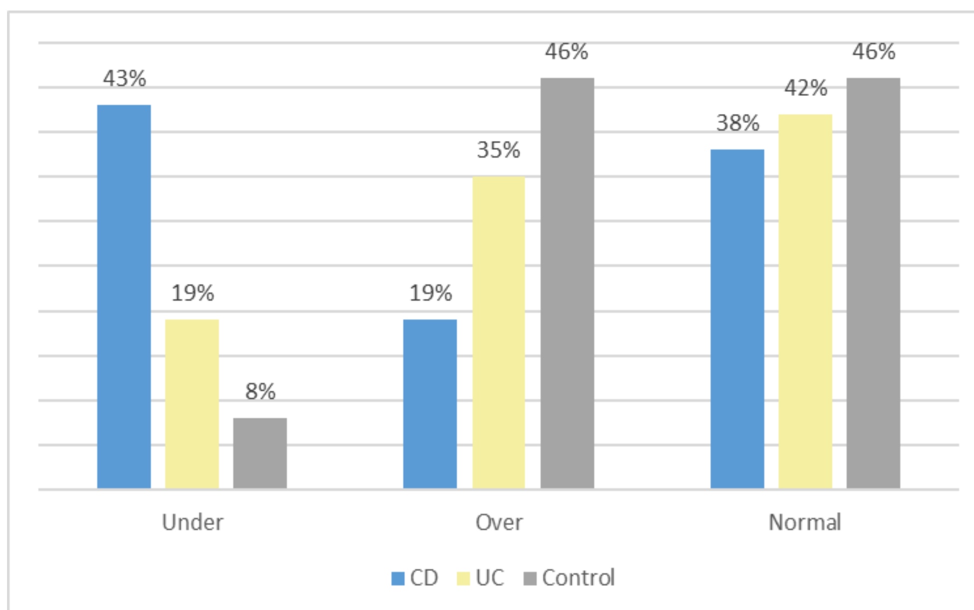


Figure 21. Differences in muscle mass among patients in the sample.

To assess the relationship between screening tools and BIA, a correlation analysis was used. In Table 22 the correlation of BIA values and the results assessed by MUST and NRS2002 are shown. A weak negative correlation between BIA results (including patient weight, BMI, and % visceral fat) and MUST score was found. The NRS2002 Screening tool did not show any significant relationship with BIA analysis (p >0.50).

Table 22. Comparison of BIA values and malnutrition screening scores (NRS2002, MUST).

BIA / Scale	Weight		SLM		BMI		Visceral fat	
	rho	p	rho	p	rho	p	rho	p
NRS2002	-0.133	>0.050	-0.083	>0.050	-0.184	>0.050	-0.198	>0.050
MUST	-0.305	0.003	-0.224	0.019	-0.329	<0.001	-0.351	<0.001

Spearman's correlation coefficient was used for the analysis

BIA, bioelectrical impedance analysis; BMI, body mass index; SLM, soft lean mass

Data obtained by BIA was compared to the control group. Statistically significant differences were observed in BMI, %visceral fat, body fat (kg), and hip-waist ratio. Other values also showed differences, but these were not statistically significant.

Analysis of body composition demonstrated considerably lower BMI values (p = 0.014) and % visceral fat mass (p = 0.003) in CD patients than in the control. Patients with UC did not show any statistically significant reduction of values compared to the control group. In contrast, patients with UC showed higher FM in kg (p = 0.046) and increased waist-hip ratio (p = 0.011).

Table 23. Comparison of parameters between the study groups and the control group

	UC	CD	Control	p-value
One-way ANOVA				
	Mean ± SD			
Weight	75.8 ± 15.1	68.7 ± 14.8	73.5 ± 13.9	0.232
PBF (%)	24.1 ± 9.9	21.3 ± 7.5	21.8 ± 9.9	0.355
Muscle mass deviation from normal	1.58 ± 4.4	0.6 ± 3.1	2.9 ± 4.6	0.143
Fat deviation from normal	4.8 ± 9.2	1.3 ± 6.8	1.8 ± 5.0	0.122
Kruskal–Wallis test				
	Median (IQR)			
Muscle mass	53.2 (44.0–60.8)	49.6 (41.1–57.3)	55.8 (42.9–61.4)	0.425
Proteins	41.7 (34.4–47.2)	38.7 (32.2–44.4)	12.5 (9.6–13.8)	<0.001
Minerals	4.30 (3.8–5.0)	3.8 (3.4–4.7)	4.3 (3.6–4.7)	0.212
TBW	41.7 (34.4–47.2)	38.7 (32.20–44.35)	43.1 (33.4–47.4)	0.454
Basal metabolism	1469.5 (1240.8–1643.0)	1503.0 (1196.00–1607.0)	1518.5 (1266.5–1711.8)	0.407
Weight deviation from normal	8.1 (-1.1–19.7)	4.1 (-8.4–6.3)	4.2 (-1.2–12.2)	0.102
Mann-Witney U test, pair-matched analysis				
	Median (IQR)			
WHR	0.9 (0.8–0.9)		0.8 (0.7–0.8)	0.011
TBF (kg)	17.8 (12.3–25.9)		14.5 (11.2–18.6)	0.046
BMI		23.4 (19.2–23.3)	21.1 (21.5–25.8)	0.041
Visceral fat %		-4.0 (-12.1–5.6)	7.9 (-0.9–18.2)	0.014

High and low disease activity showed significant differences across nutritional screening scales (Table 24). Patients with a high activity index had a noticeably increased risk for malnutrition, taking into consideration not only disease activity but also increased weight loss and loss of appetite.

Table 24. Comparison of high- and low-disease activity groups

Score	High activity	Low activity	*P value*
NRS2002	0.30 (2.00–4.00)	0.00 (0.00–2.00)	0.007
MUST	2.00 (1.00–3.00)	0.00 (0.00–1.00)	<0.001

DISCUSSION

The frequency of IBD is increasing and the diagnosis and treatment of IBD patients is a problem in Latvia and worldwide. Early diagnosis and personalised treatment are vital for IBD patients to improve their quality of life, reduce the risks of disability and oncology, and reduce the risk of treatment complications. In Latvia, the rapid spread of IBD is a concern for health care professionals and the public, as this diagnosis is increasingly common for younger patients of working age. In our study, almost half of the IBD patients were over 40 years of age. This means that in the future, the country will have an ageing population of IBD patients with complicated comorbidities and an increased risk of thiopurine side effects.

To assess the toxicity of thiopurine drugs and to select the optimal dose, TPMT enzyme activity should be assessed by identifying the most common polymorphisms affecting TPMT activity in all patients receiving thiopurine therapy. Past studies have recommended that the TPMT status of patients should be determined prior to the commencement of thiopurine therapy (Benmassaoud et al., 2016; Liu et al., 2015; Lennard, 2014; Liu et al., 2016; Coelho et al., 2016). This can be achieved by one of two methods: 1) by determining the TPMT phenotype by estimating TPMT enzyme activity in the circulating red blood cells, or 2) through genotyping of known TPMT variants associated with enzyme deficiency using PCR (Liu et al., 2016; Coelho et al., 2016; Goel et al., 2016). The activity of the TPMT enzyme is mainly related to rs1800462, rs1800460, and rs1142345 SNPs, which are inherited co-dominantly (Lennard, 2014; Asadov et al., 2017). In terms of AZA toxicity, large inter-individual differences are observed due to the genetic heterogeneity of the TPMT gene, which has more than 40 reported allelic variants (Coelho et al., 2016; Dean, 2012). Nevertheless, three main patterns of TPMT enzyme activity can generally be distinguished: 1) homozygous patients with two mutant non-functional TPMT gene alleles and low TPMT activity, 2) heterozygous individuals with one functional and one non-functional allele and intermediate TPMT activity, and 3) homozygous wild-type (normal) individuals with two functional alleles and normal or high TPMT activity (Lennard, 2014; Asadov et al., 2017; Dean, 2012; Chouchana et al., 2014).

Approximately 40 TPMT gene polymorphism variants associated with decreased TPMT activity have been described in the literature. However, only the four most common alleles, TPMT * 2, TPMT * 3A, TPMT * 3B and TPMT * 3C, are used for most genotyping tests (Asadov et al., 2017; Dean, 2012). Although known TPMT alleles tend to vary among different ethnic groups, four specific non-functional alleles have been identified as being more prevalent across ethnic groups, namely, TPMT * 2, TPMT * 3A, TPMT * 3B, and TPMT * 3C. It is believed that these alleles account for between 80% and 95% of observed decreases in TPMT enzyme activity. Accordingly, these four alleles tend to be routinely targeted in most of the genotyping assays (Skrzypczak-Zielinska, 2016; Asadov et al., 2017; Dean, 2012). In general, the most frequently encountered allele in all populations

is TPMT * 3A, followed by TPMT * 3C (Carvalho et al., 2014; Fangbin et al., 2016; Lennard, 2014), which is consistent with the findings of the present study. To our knowledge, this study is the first to identify TPMT gene polymorphisms in adult IBD patients in Latvia.

The most common TPMT genotype in Caucasian populations is the homozygous, wild-type TPMT gene. Several studies have shown that 85–95% of patients have a wild-type genotype (93.9% in this study). In most populations, approximately 10% of individuals are heterozygotes and a further 0.3% carry homozygous variants of the non-functional TPMT alleles (Asadov et al., 2017; Dean, 2012; Broekman et al., 2017; Gonzalez-Lama and Gisbert, 2016). In the present study, none of the participating patients was identified as being homozygous for any mutation. TPMT genotyping has been demonstrated to show high sensitivity (Almoguera et al., 2014; Gonzalez-Lama and Gisbert, 2016), with reported sensitivities and specificities of 88.9% (81.6–97.5%) and 99.2% (98.4–99.9%), respectively. Comparatively, the approximate sensitivity and specificity of TPMT phenotyping are 91.3% (86.4–95.5%) and 92.6% (86.5–96.6%), respectively (Goel et al., 2016).

In our study, 9% of IBD patients were undergoing thiopurine therapy at the time of their inclusion. This percentage does not include patients who had used thiopurines previously but had since stopped. Indeed, most patients included in the study had not yet started thiopurine treatment.

In 2015, the US FDA issued guidelines for TPMT testing prior to thiopurine therapy due to the increased risk of toxicity and high treatment costs. TPMT analysis can be performed by determining TPMT enzyme activity or by genotyping and allows doctors to identify an optimised starting dose of thiopurines, as well as alternative therapies in case of thiopurine toxicity. One major advantage of TPMT genotyping is that polymorphisms can be detected both before and during thiopurine therapy. While TPMT enzyme activity can be affected by many external factors, such as previous haemotransfusions, other drug therapies, and drug interactions, genotyping is not affected by these factors as DNA is much more stable. The disadvantage of TPMT genotyping is that this method cannot identify patients with elevated TPMT enzyme activity. According to the literature, increased TPMT enzyme activity, which can be determined by liquid chromatography, may increase the risk of developing hepatotoxicity (Ribaldone et al., 2019; Coenen et al., 2015).

With regard to the cost-effectiveness of genotype-led treatment, recent data published by Sluiter et al. (2019) showed that genotype-guided thiopurine treatment in IBD patients reduced the risk of adverse drug reaction (ADR) among patients carrying a TPMT variant, without increasing overall healthcare costs and impacting the quality of life compared to standard treatment.

The Clinical Pharmacogenetics Implementation Consortium (CPIC) published recommendations for AZA dosing based on TPMT genotyping results on AZA use, stressing the need to consider a dose reduction of AZA or other classes of drug treatment in patients with low or insufficient TPMT activity (Sluiter et al., 2019).

In patients with two functionally active TPMT alleles, the activity of the TPMT enzyme is in most cases normal or high. For these patients, the CPIC recommends initiating AZA therapy at a normal dosage and thereafter adjusting the dose based on disease-specific guidelines. In heterozygous patients, TPMT enzyme activity is moderate, and these patients are at an increased risk of AZA-induced myelosuppression, depending on the dose. Accordingly, the CPIC recommends that for heterozygous patients, AZA treatment should be initiated at 30–70% of the target dose and to titrate the dose based on tolerance. For homozygous patients with two non-functional TPMT alleles and consequently low TPMT enzyme activity, the use of alternative treatment is suggested (Frei et al., 2013; Dean, 2012) Interestingly, in this regard, a previous study that sought to determine a safe dosage of AZA in patients with intermediate or low TPMT enzyme activity, found that TPMT genotyping could facilitate a reduction in adverse haematological effects of up to 89% (Freuerstein et al., 2017).

Given that TMPT can vary quite extensively depending on age, sex, ethnicity, red blood cell lifespan, red blood cell transfusion, leukaemia, and treatment-related factors, these factors should be considered when interpreting TPMT activity analysis (Asadov et al., 2017; Chouchana et al., 2014). In addition, assessments of TPMT activity may require repeated estimations, as treatment with AZA can induce an increase in TPMT activity. Similarly, other medications, such as 5-aminosalicylates, can reversibly inhibit TPMT activity (De Boer et al., 2007; Glissen et al., 2005). Therefore, TPMT genotyping is considered a more precise and reliable method (Frei et al., 2013). Typically, the overall concordance between TPMT genotypes and phenotypes is 90–95%, although some studies have reported genotype–phenotype concordance values of approximately 60–70% in patients with low TPMT activity, whereas those for heterozygous patients are approximately 70–86% (Lennard, 2014; Asadov et al., 2017).

However, even though the identification of TPMT genotypes and/or phenotypes can contribute to identifying patients with a higher risk of developing bone marrow toxicity, additional therapeutic drug monitoring is advised for patients receiving AZA therapy (Yarur et al., 2014). Some authors recommend monitoring complete blood counts (CBC) and (platelet counts) PC using routine laboratory tests at weekly intervals during the first month of AZA treatment, followed by twice-monthly monitoring during the second and third months, and monthly checks thereafter. Furthermore, liver function tests should be performed at 3-month intervals (Goel et al, 2015). If, however, signs of myelosuppression develop, AZA therapy should be immediately discontinued.

A recent publication by Ribaldone et al. (2019) titled 'Correlation between Thiopurine S-Methyltransferase Genotype and Adverse Events in Inflammatory Bowel Disease Patients' Medicina (Kaunas), described a meta-analysis investigating the associations between TPMT polymorphisms and AZA-induced adverse events in patients with autoimmune diseases. The results showed that TPMT polymorphisms were significantly associated with AZA-induced adverse effects, bone marrow toxicity, and gastric intolerance. However, the subgroup analysis according to ethnicity showed a

significant association between TPMT polymorphisms and AZA-induced bone marrow toxicity in Asian populations, but not in Caucasian populations. The authors concluded that TPMT polymorphisms can explain a variable proportion, but not all, of AZA-related adverse events, and a normal TPMT genotype cannot exclude the development of side effects (Ribaldone et al., 2019). Thus, TPMT genotyping before starting AZA therapy cannot completely replace the current practice of periodic monitoring of white blood cell count. However, this is a challenge that requires additional future research, particularly as some severe toxicities leading to life-threatening conditions remain unexplained.

It is important to note that the randomised controlled trial conducted by Coenen et al. (2015) showed that pretreatment TPMT genotyping is relevant for both heterozygous and homozygous carriers of genetic variants in TPMT (Coenen et al., 2015). The results of the trial showed no overall effect of pretreatment TPMT screening followed by personalised dosing on hematologic ADRs. However, in combination with other literature, this study showed that pretreatment TPMT screening followed by personalised dosing reduced the risk of leukopenia in patients carrying a genetic variant in TPMT and recommended that pharmacogenetic TPMT testing should be used as standard care to individualise the thiopurine treatment of IBD patients (Coenen et al., 2015) Thiopurines remain very effective in inducing and maintaining long term remission in up to 70% of patients with IBD, and it is important to remember that patients with allelic variants should not be denied the therapeutic option of AZA, as they may tolerate this drug (Ribaldone et al., 2019).

In our study, 98% of patients with TPMT polymorphisms were European. The frequency of mutant alleles varies in different ethnic groups is known, but in general, the most common allele globally is TPMT * 3A, followed by TPMT * 3C (Almoguera et al., 2014; Carvalho et al., 2014; Fangbin et al., 2016). In our study, we obtained similar data, with the most common allele in IBD patients being TPMT * 3A, followed by TPMT * 3C and TPMT * 2 with equal frequency. There is evidence in the literature that the TPMT * 3B allele is present in up to 1% of people, but this allele was not observed in our study.

The most common TPMT genotype is the homozygous wild-type TPMT gene, which is associated with normal TPMT enzyme activity. Several studies have shown that around 85–95% of people have two functioning alleles. This agrees with the prevalence in our study of 93.9%. Patients with this genotype can start taking thiopurines at standard doses, but can still experience thiopurine side effects due to other factors. Therefore, if thiopurines are used, blood tests, and possibly metabolites of thiopurines, should be monitored (Asadov et al., 2017; Dean, 2012),

Approximately 10% of people are heterozygous for the TPMT genotype (6.1% of patients in our study), and theoretically, have reduced TPMT enzyme activity and are at increased risk of side effects from treatment with thiopurines (Almoguera et al., 2014). In our study, no patients had a homozygous variant for any of the mutations tested, whereas the literature estimates that 0.3% of the

population have a homozygous variant of non-functional TPMT alleles (Asadov et al., 2017; Dean, 2012; Broekman et al., 2017; Gonzalez-Lama and Gisbert, 2016).

According to the literature, approximately 10–20% of IBD patients who receive AZA therapy discontinue treatment either because they develop adverse reactions or the treatment is ineffective (Liu et al., 2015; Ardizzone et al., 2004). The most common complications of AZA are AST disorders, hepatotoxicity, infections, and myelosuppression (Kim and Choe, 2013; Benmassaoud et al., 2016; Frei et al., 2013). However, the meta-analysis of Liu et al. (2015) concluded that the genetic polymorphism of TPMT is more associated with myelosuppression and KTT disorders. In our study, of the 6.1% (n = 15) of patients with TPMT polymorphisms, two of them had a history of AZA adverse events such as myelosuppression.

Almost half 43% (n = 105) of the patients in our study were smokers in their lifetime and 15% (n = 37) were current smokers, with a mean smoking duration of 14 years. The effects of smoking on the pathogenesis and recurrence of IBD are well known, but the effects of smoking on the metabolism of thiopurine drugs have been less studied. A retrospective study by Domenech et al. (2011) found that while active smoking did not affect the efficacy of thiopurines, active smokers were more likely to experience thiopurine side effects. Shi et al. (2015) found that active smoking and reduced TPMT enzyme activity were associated with higher levels of 6-TGN metabolites (Shi et al., 2015). This suggests that the interaction between smoking and thiopurine metabolism is still unclear and should be considered in patients prescribed thiopurine therapy.

From analysing the IBD patients in our study, we obtained a statistically reliable result that IBD patients remain more physically inactive after diagnosis than before the onset of the disease. Similar results have been found in previous studies. Gatt et al. (2019) found that patients were significantly less physically active after the diagnosis of IBD, and this was more common in CD patients (Gatt et al., 2019). A personalised approach and better control of disease activity would be needed to help address patients' lower physical activity and loss of quality of life.

No previous studies have been performed in Latvia to determine the frequency of TPMT gene polymorphisms in the population, as well as among IBD patients specifically. This study is the first in Latvia to identify TPMT gene polymorphisms in adults diagnosed with IBD. In the future, TPMT status should be determined in clinical practice in IBD patients who need to start thiopurines treatment to avoid serious and life-threatening complications, as well as to predict the effectiveness of treatment.

Malnutrition can be subdivided into several subtypes, depending on the causative factor. Due to the many possible causes, there is a wide definition of malnutrition and undernutrition, making it complicated to determine a diagnosis (Cederholm et al., 2017). As it is important to establish an accurate nutritional status for patients, numerous nutritional screening tools have been developed; however, none is considered the gold standard for nutritional assessment (Ghishan and Kiela, 2017). Both screening tools used in this study (NRS2002 and MUST) are recommended by EPSEN guidelines

(Kondrup, 2002). There was strong agreement between both tools in terms of evaluating the nutritional status of the patients. This is similar to the findings of Raupp et al. (2018). Nutritional assessment was performed 48 h after admission to the hospital using NRS2002 and MUST. Both results were compared to a subjective global assessment (SGA) and presented a good agreement in nutritional status evaluation (Raupp et al., 2018). However, NRS2002 is more specific due to scaling disease activity, while MUST establishes patients with severe disease by defining them as 'high nutritional risk'; therefore, it may overestimate nutritional risk. Another possible reason for higher scores obtained by MUST is that this scale evaluates patients over a longer period. Thus, if weight loss is gradual, for example, the patient showed a weight loss of over 5% over the previous 3 months, no points will be given by NRS2002. Indeed, in our study, two patients lost weight over a longer period and received points by the MUST scale but not NRS2002.

A previous study by Valentini et al. (2008) evaluated the nutritional status of IBD patients. They found that patients in remission who seemed well-nourished by screening tools tended to have reduced body cell mass, reduced handgrip strength, and micronutrient deficits. Their results indicated that nutritional deficit occurs in the same ratio for UC and CD patients, in contrast to our data which showed that CD patients were slightly more affected (Valentini et al., 2008). Several studies agree with the present findings that CD patients are at a higher nutritional risk than UC patients, even among those in clinical remission (Ghishan and Kiela, 2017; Jahnsen, 2003; Ananthakrishnan et al., 2012). This might be explained by the involvement of the small bowel, which leads to impaired absorptive function and loss of nutrients due to fistulas (Rocha et al., 2008). Chronic inflammatory processes and lack of physical activity stimulate muscle deterioration, leading to sarcopenia (Cederholm, 2017). In our study, patients had several micronutrient deficits, but no statistical relationship with screening tools was found. Patients who appear to be in clinical remission and without signs of undernutrition may still have micronutrient deficits.

Patients with UC showed better body composition parameters than CD patients. Sarcopenia in UC patients is more dependent on disease activity, where the change in muscle mass correlates with an increase in the Mayo score. Patients in remission show better body composition values, as did UC patients after colectomy (Zhang et al., 2017).

Most patients had a BMI within normal values, however, in many cases, a disproportion of body composition was observed. The latter highlights the need for a closer investigation of the patient's nutritional status, rather than relying solely on BMI. It has been reported that BMI correlates with the FMI, therefore BMI is better at predicting body fat mass than muscle mass (Bryant et al., 2013).

Not all patients screened to be at nutritional risk received clinical feeding. This was determined at the physician's discretion, as some patients who were able to eat nutritionally rich food and whose disease activity was controlled adequately with medical therapy were not deemed to require additional

feeding. Patients who received nutritional support without being in a high-risk group were previously identified as ‘nutritionally at risk’; thus, they continued to receive nutritional support as a part of treatment. Nutritional management should be defined depending on the patient’s nutritional status, and considering the requirements of energy and nutrients, appropriate route of administration to adequately set goals, and duration of treatment to achieve them (Cederholm, 2017).

The BIA showed a statistically significant correlation with the MUST scale and more significant changes in body composition were observed in CD patients. Among patients with normal BMIs, imbalances in body composition can be observed, stressing the importance of broader body composition analysis.

CONCLUSIONS

1. Our results showed that the frequencies of common TPMT alleles in the Latvia IBD population were different (similar to other European populations). In this study, the homozygous wild-type TPMT *1/*1 genotype was the most frequent genotype in UC and CD patients and TPMT * 3A was the most prevalent polymorphism. Further, TPMT * 3B polymorphism and homozygous variant TPMT genotypes were absent in our study population.
2. A majority of patients had normal TPMT phenotype, as few of patients had low TPMT activity (10%) and 15% of patients were identified as hyperactive metabolizers. This pilot study is a first published in Baltic states that introduced the enzyme-linked immunosorbent assay method for assessment of TPMT enzyme activity in blood.
3. IBD patients with a high disease activity index were at a noticeably increased risk of malnutrition, considering not only IBD activity but also weight loss and loss of appetite. Most CD patients showed a reduction in muscle mass in both groups with low and high disease activity, which was not found in UC patients.

PRACTICAL RECOMMENDATIONS

1. We recommend that TPMT genotyping or phenotyping should be prioritised for higher-risk patients to help predict thiopurine-induced adverse drug reactions and to determine personalised therapeutic options. Additional genotyping of patients experiencing adverse effects due to thiopurine treatment will be required to identify potential gene/allele–dose effects.
2. Identification of reduction in muscle mass (soft lean muscle mass*)* in CD patients can be considered as an anticipatory indicator of disease activity.

PUBLICATIONS AND THESIS OF THE AUTHOR

Latvian patent

Latvijas Republikas patentu valdes oficiālais izdevums 2/2021

The Official Gazette of the Patent Office of the Republic of Latvia – 'Izgudrojumi, Preču Zīmes un Dizainparaugi' - February 20, 2021.

ISSN 2255-9655

Number of the patent: Nr. LV 15508 B1

Indication of International Patent Classification: C12Q 1/6827; C12Q 1/48; C12Q 1/686

Patent name: Method of detection of thiopurine methyltransferase polymorphisms

Name(-s) of inventors(-s): Aldis Puķītis (LV), Poļina Zaļizko (LV), Juris Stefanovičs (LV), Jeļizaveta Sokolovska (LV)

Patent publication 20.02.2021

Publications in cited and peer-reviewed journals

1. Zalizko P, Roshofa TH, Meija L, Bodnieks E, Pukitis A. The role of body muscle mass as an indicator of activity in inflammatory bowel disease patients. Clinical Nutrition European Society for Clinical Nutrition and Metabolism Journal, 2020, 40: 193-200. https://doi.org/10.1016/j.clnesp.2020.09.023
2. Zalizko P, Stefanovics J, Sokolovska J, Paramonova N, Klavina E, Erts R, Rovite V, Klovins J, Pukitis A. Thiopurine S-methyltransferase genetic polymorphisms in adult patients with inflammatory bowel diseases in the Latvian population. Therapeutic Advances in Gastroenterology, 2020, 13: 1-8. https://doi.org/10.1177/1756284820937426
3. Zalizko P, Jargane I, Pukitis A. Therapeutic drug monitoring of thiopurine therapy in patients with inflammatory bowel disease. Japanese Journal of Gastroenterology and Hepatology, 2019, 1(4): 1-4. https://doi.org/10.35665/2435-1210.2019.1017
4. Zalizko P, Urbane M, Roshofa TH, Mokricka V, Meija L, Bodnieks E, Pukitis A. Body muscle mass metabolic data analysis in association with Crohn's disease activity. Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences, 2022, 76, 1(736): 17-21. https://doi.org/10.2478/prolas-2022-0003

Other publications

1. Zaļizko P, Jargāne I, Mokricka V, Dzirkale Z, Popēna I, Narbute K, Jirgensone T, Tropiņa E, Beitnere U, Puķītis A. Azatioprīna terapijas riska izvērtēšana pacientiem ar iekaisīgām zarnu slimībām, pielietojot tiopurīna metiltransferāzes enzīma ekspresijas noteikšanas metodi. LU Raksti (Scientific Papers University of Latvia). Medicine. 2021. g. *(accepted for publication)*
2. Zalizko P, Tropina E, Scholbach T, Puķītis A. Association of Dunbar, May-Thurner and Nutcracker Compression Syndromes in One Patient. Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences 2020, 74(2): 150-155. https://doi.org/10.2478/prolas-2020-0024
3. Zalizko P, Stefanovics J, Erts R, Rovite V, Klovins J, Pukitis A. Unexplained higher frequency of mutant thiopurine S-methyltransferase genotypes in inflammatory bowel disease patients of Latvia population, Journal of Crohn's and Colitis, 2019, 13(1): S537, https://doi.org/10.1093/ecco-jcc/jjy222.950
4. Zalizko P, Mokricka V, Jargane I, Tropina E, Dzirkale Z, Popena I, Narbute K, Jirgensone T, Beitnere U, Pukitis A. Thiopurine therapy risk evaluation for inflammatory bowel disease patients using TPMT enzyme expression method. American Journal of Gastroenterology, 2017, 112: 1484. https://doi.org/10.1038/ajg.2017.336
5. Skuja V, Pekarska K, Straume Z, Rudzīte D, Lavrinoviča E, Piekuse L, Dauvarte H, Vašuka E, Dobelniece L, Zalizko P, Derovs A, Krūmiņa A, Lejnieks A. Ciprofloxacin resistance in ESBL producing enterobacteriaceae colonizing the gut in IBD patients. Journal of Crohn's and Colitis, 2017, 11(1): S481, https://doi.org/10.1093/ecco-jcc/jjx002.904
6. Dauvarte H, Vašuka E, Dobelniece L, Zaļizko P, Skuja V, Derovs A, Lejnieks A. Antibakteriālā terapija čūlainā kolīta un Krona slimības pacientiem: sešu gadu pieredze divas Latvijas universitātes slimnīcās. RSU Zinātniskie raksti 2017, 98-105.
7. Skuja V, Pekarska K, Straume Z, Dauvarte H, Rudzīte D, Lavrinoviča E, Piekuse L, Vašuka E, Dobelniece, Zalizko P, Derovs A, Lejnieks A, Krūmiņa A. Current use of antibacterial agents may act as a risk factor for gut colonization with ESBL producing Enterobacteriaceae in Ulcerative Colitis patients. Proceedings of the 59th International Scientific Conference of Daugavpils University, Part A. Natural Sciences, 2017, 520-531.

Thesis and presentations in international conferences

1. Zalizko P, Urbane M, Roshofa TH, Mokricka V, Meija L, Bodnieks E, Pukitis A. Body muscle mass association with Crohn's disease activity. International Scientific Conference on Medicine at University of Latvia, Riga, Latvia, April 23-24, 2021.

2. Prohorova N, Zaļizko P. Change of diet habits in Inflammatory bowel disease patients after diagnosis – a prospective study. Rīga Stradiņš University International student conference, Riga, Latvia, March 22-23, 2021, p.134.
3. Zalizko P, Roshofa TH, Mokricka V, Meija L., Bodnieks E., Pukitis A. Analysis of body muscle mass in Crohn's disease patients. The 3rd International Conference Nutrition and Health, Riga, Latvia, December 9–11, 2020, p. 90.
4. Zalizko P, Stefanovics J, Mokricka V, Rovite V, Klovins J, Pukitis A. Genetic testing of HLA-DQA1-HLA-DRB1 in patients with IBD. United European Gastroenterology Week 2020, October 11–13, 2020.
5. Zalizko P, Stefanovics J, Mokricka V, Rovite V, Klovins J, Pukitis A. Thiopurine monitoring in late-onset inflammatory bowel disease patients. Medicina (Kaunas) 2020; 56 (Supplement 1): 28. 78th Scientific Conference of the University of Latvia, International Medical Section. Riga, 2020.
6. Zalizko P, Stefanovics J, Erts R, Rovite V, Klovins J, Pukitis A. Use of TPMT genotyping for evaluation of thiopurines toxicity risk in late-onset IBD patients. United European Gastroenterology Week 2019, Barcelona, Spain, October 21–23, 2019, p. 603.
7. Zalizko P, Roshofa TH, Meija L, Pukitis A. Malnutrition risk screening in hospitalized patients with IBD. Falk Symposium 216, Brussels, Belgium, September 13–14, 2019, p. 87.
8. Tropina E, Sauša L, Zalizko P. Evaluation of two different fecal calprotectin detection methods used in Pauls Stradins Clinical University Hospital. 15th Warsaw international medical congress, Warsaw, Poland, May 9-12, 2019, p. 123.
9. Zalizko P, Mokricka V, Pokrotnieks J, Pukitis A. Persistent fever – rare presentation of antiTNF induced systemic reaction in patient with severe Crohn's disease. Falk Symposium 214, Inflammatory Bowel Disease: From Pathophysiology to Personalized Medicine, Oxford, Great Britain, March 29–30, 2019, p. 101.
10. Zalizko P, Mokricka V, Pukite I, Sergejeva J, Pokrotnieks J, Pukitis A. Infliximab therapy and tight disease monitoring during pregnancy for Crohn's disease patient with high disease activity. Falk Symposium 214, Inflammatory Bowel Disease: From Pathophysiology to Personalized Medicine, Oxford, Great Britain, March 29-–30, 2019, p. 51.
11. Zalizko P, Pukitis A. Perspectives of TPMT genotyping. Baltic Countries IBD Experts meeting, Riga, Latvia, March 29, 2019.
12. Zalizko P, Stefanovics J, Erts R, Rovite V, Klovins J, Pukitis A. Unexplained higher frequency of mutant TPMT genotypes in IBD patients of Latvia population. European Crohn's and Colitis Organisation Congress, Inflammatory Bowel Diseases 2019, Copenhagen, Denmark, March 6–9, 2019.
13. Zalizko P, Stefanovics J, Sokolovska J, Paromonova N, Erts R, Mokricka V, Rovite V, Klovins J, Pukitis A. The prevalence of polymorphisms of thiopurine methyltransferase gene in Latvian

population of inflammatory bowel disease patients. International Scientific Conference on Medicine at University of Latvia, Riga, Latvia, February 22, 2019, p. 50.

14. Roshofa TH, Zalizko P, Meija L, Pukitis A. The assessment of malnutrition in inflammatory bowel disease patients. International Scientific Conference on Medicine at University of Latvia, Riga, Latvia, February 22, 2019, p. 146.
15. Zalizko P, Stefanovics J, Erts R, Rovite V, Klovins J, Pukitis A. TPMT genotype polymorphism of selected IBD population in Latvia. World Gastroenterology Organisation International Conference, Global Perspectives in Gastroenterology, Bangkok, Thailand, December 5–8, 2018, p.50.
16. Zalizko P, Mokricka V, Jargane I, Tropina E., Servinska Z., Pukitis A. Jaunas diagnostiskas metodes ieviešana tiopurīnu terapijas monitoringā pacientiem ar IZS. IV Pasaules latviešu zinātnieku kongress. Rīga, Latvija, June 18–20, 2018, p.14.
17. Zalizko P, Mokricka V, Pavars M, Pokrotnieks J, Pukitis A. Spleen abscess. Rare complication of Crohn's disease. Falk Symposia (210), Lisbon, Portugal, April 20–21, 2018, p. 174.
18. Zalizko P, Mokricka V, Pokrotnieks J, Pukitis A. Acne vulgaris related to ulcerative colitis treated with infliximab standard therapy. Falk Symposia (210), Lisbon, Portugal, April 20–21, 2018, p.109.
19. Tropiņa E, Sauša L, Zalizko P. Comparison of enzyme linked immunosorbent assay and rapid test used in fecal calprotectin detection. Rīga Stradiņš University International student conference, health and social sciences. Riga, Latvia, March 16-17, 2018, p.38.
20. Zalizko P, Pukitis A. The evaluation of azathioprine therapy risk for patients diagnosed with inflammatory bowel disease. 76th Scientific Conference of the University of Latvia, International Medical Section. Riga, February 21, 2018, p.54.
21. Zalizko P, Pukitis A. "Safety of thiopurines therapy in patients with late-onset inflammatory bowel disease" European Crohn's and Colitis Organisation Congress, Inflammatory Bowel Diseases 2018, Vienna, Austria, February 14, 2018.
22. Zalizko P, Pukitis A. "The evaluation of azathioprine therapy risk for IBD patients" BIT's 2nd Annual World Congress of Digestive Disease, Fukuoka, Japan, December 4–6, 2017, p. 101.
23. Zalizko P, Mokricka V, Pukitis A. Thiopurine S-Methyltransferase activity analysis in patients with inflammatory bowel diseases. Falk Symposia (209), Berlin, Germany, October 6–7, 2017, p. 147.
24. Skuja V, Pekarska K, Straume Z, Dauvarte H, Rudzīte D, Lavrinoviča E, Piekuse L, Vašuka E, Dobelniece L, Zalizko P, Derovs A, Lejnieks A, Krūmiņa A. Current use of antibacterial agents may act as a risk factor for gut colonization with ESBL producing Enterobacteriaceae in Ulcerative Colitis patients. 59th International Scientific Conference of Daugavpils University, Latvija, April 6-7, 2017, p. 47.

25. Skuja V, Pekarska K, Straume Z, Dauvarte H, Rudzīte D, Lavrinoviča E, Piekuse L, Kempa I, Goida E, Dobelniece L, Zalizko P, Derovs A, Lejnieks A, Krūmiņa A. Ciprofloxacin resistance in ESBL producing Enterobacteriaceae colonizing the gut in IBD patients. 12th Congress of ECCO – Inflammatory Bowel Diseases, Barcelona, Spain, February 15-18, 2017.

Thesis and presentations in local conferences

1. Zaļizko P. Azatioprīna loma mūsdienu IZS ārstēšanas stratēģijā. Gastroenteroloģijas Vasaras skola 2020, Rīga, Latvija, 4.-5. septembris, 2020.
2. Zaļizko P. Personalizētā medicīna: Mūsdienu tehnoloģiju ieviešana, ārstējot individualizēti. Dienas bizness Medicīnas aprūpes nozares konference: Kad rītdiena ir šodiena – aktuālās tendences medicīnas aprūpē un farmācijā, Rīga, Latvija, 2019.g. 6. jūnijs.
3. Zaļizko P., Jargāne I. "Azatioprīna terapijas riska izvērtēšana pacientiem ar iekaisīgām zarnu slimībām, pielietojot TPMT enzīma ekspresijas noteikšanas metodi" Latvijas Farmaceitu biedrības "LFB Gada konferencē 2017" Latvijas Universitātes Dabaszinātņu akadēmiskajā centrā 09.01.2018.

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisor and mentor – Professor Aldis Puķītis. Many thanks to professor Puķītis for his support and patience throughout the dissertation process. It was a challenging path of scientific research, but the professor always supported my scientific work and even when things did not go as planned, he always supported and motivated me. I am grateful for his valuable guidance, help during the preparation of this dissertation, and an opportunity to combine my passion for science with the workplace.

I would like to further acknowledge my colleagues from Gastroenterology, Hepatology and Nutrition centre in Pauls Stradins Clinical University Hospital. Thanks to all doctors, residents and nurses in Gastroenterology Department No. 10, and special thanks to Dr Viktorija Mokricka for her help and advice, Dr med. Aiga Stāka, Dr Ēvalds Ostrovskis, Dr Jeļena Ivanova, Dr Anda Altberga, Dr Edgars Bodnieks, and Dr med. Laila Meija for their moral support and advice when needed. Thanks to Professor Juris Pokrotnieks for inspiring me in my personal and professional development. Thanks to Tatjana Jirgensone – a head nurse of the Gastroenterology department and her staff for helping in practical work. I am grateful to my all students for practical help in data collection and compilation.

I am very appreciating for all the Laboratory of Personalized Medicine, Faculty of Medicine, University of Latvia. Special thanks for Dr med. Jeļizaveta Sokolovska for her attentiveness, constructivism and support. I would like to express sincere thanks to my colleague Juris Stefanovičs without whom I could not have completed this work. Sincere thanks to Dr biol. Natalia Paramonova for helping in practical experiments and her expert advice.

I am grateful to the Latvian Biomedical Research and Study Centre for practical support, especially Dr biol. Vita Rovīte and Dr biol., Professor Jānis Kloviņš for responsiveness and encouragement for completing my work.

Thanks to colleagues from the Department of Pharmacology, Faculty of Medicine, University of Latvia, especially Dr pharm. Zane Dzirkale, Ineta Popēna, Karīna Narbute, Ulrika Beitnere, and Inese Jargāne for their help in polymerase chain reaction experiments and advice.

Many thanks to Dr med. Renārs Erts for his help in the selection of statistical methods for research and data processing.

Special thanks to the University of Latvia and Faculty of Medicine for the valuable doctoral studies and financial support in publishing my research.

I am also thankful to the University of Latvia and the University of Latvia Foundation for the opportunity to receive a stipend from SIA 'Mikrotīkls' that was a great financial support in publishing this research. Thanks to SIA 'Mikrotīkls'.

I express my gratitude to my family, my parents Inna and Sergej Zalizko, who always gave me a moral support, love and encouragement. They always believed in me and even in times of doubt, gave me strength and confidence that everything will work out. Special thanks to my brother Nikita and my sisters Sofja and Faina. Thanks to my husband Vladislav Suharevs for your patience, understanding and support throughout the work.

I express my gratitude to all those people who helped me complete this dissertation work!

REFERENCES

1. Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med. 2009; 361(21): 2066-78.
2. Almoguera B, Vazquez L, Connolly JJ, Bradfield J, Sleiman P, Keating B, Hakonarson H. Imputation of TPMT defective alleles for the identification of patients with high-risk phenotypes. Front Genet. 2014; 5: 96.
3. Ameyaw MM, Collie-Duguid ES, Powrie RH, Ofori-Adjei D, McLeod HL. Thiopurine methyltransferase alleles in British and Ghanaian populations. Hum Mol Genet. 1999; 8(2): 367-70.
4. Ananthakrishnan A. Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol 2015; 12: 205–217.
5. Ananthakrishnan AN, Khalili H, Higuchi LM, Bao Y, Korzenik JR, Giovannucci EL, Richter JM, Fuchs CS, Chan AT. Higher predicted vitamin D status is associated with reduced risk of Crohn’s disease. Gastroenterology 2012; 142: 482-489.
6. Ardizzone S, Maconi G, Sampietro GM, Russo A, Radice E, Colombo E, Imbesi V, Molteni M, Danelli PG, Taschieri AM, Bianchi Porro G. Azathioprine and mesalamine for prevention of relapse after conservative surgery for Crohn’s disease. Gastroenterology. 2004; 127(3): 730-40.
7. Argilés JM, Busquets S, Stemmler B, López-Soriano FJ. Cachexia and sarcopenia: mechanisms and potential targets for intervention. Curr Opin Pharmacol. 2015; 22: 100-106.
8. Armstrong VW and Oellerich M. New developments in the immunosuppressive drug monitoring of cyclosporine, tacrolimus, and azathioprine. Clin Biochem. 2001; 34: 9–16.
9. Asadov C, Aliyeva G and Mustafayeva K. Thiopurine S-Methyltransferase as a pharmacogenetic biomarker: Significance of testing and review of major methods. Cardiovasc Hematol Agents Med Chem. 2017; 15: 23–30.
10. Back IR, Marcon SS, Gaino NM, Vulcano DSB, Dorna MS, Sassaki LY. Body composition in patients with Crohn’s disease and ulcerative colitis. Arq Gastroenterol. 2017; 54(2): 109-114.

11. Benmassaoud A, Xie X, AlYafi M, Theoret Y, Bitton A, Afif W, Bessissow T. Thiopurines in the management of Crohn's disease: safety and efficacy profile in patients with normal TPMT activity-A retrospective study. Can J Gastroenterol Hepatol. 2016; 2016: 1034834.
12. Best WR. Predicting the Crohn's disease activity index from the Harvey-Bradshaw Index. Inflamm Bowel Dis. 2006; 12(4): 304-10.
13. Bischoff SC, Escher J, Hébuterne X, Kłęk S, Krznaric Z, Schneider S, Shamir R, Stardelova K, Wierdsma N, Wiskin AE, Forbes A. ESPEN practical guideline: Clinical nutrition in inflammatory bowel disease. Clin Nutr. 2020; 39: 632-653.
14. Blaker PA, Arenas-Hernandez M, Smith MA, Shobowale-Bakre EA, Fairbanks L, Irving PM, Sanderson JD, Marinaki AM. Mechanism of allopurinol induced TPMT inhibition. Biochem Pharmacol. 2013 Aug; 86(4): 539-547.
15. Broekman MMTJ, Coenen MJH, Wanten GJ, van Marrewijk CJ, Klungel OH, Verbeek ALM, Hooymans PM, Guchelaar HJ, Scheffer H, Derijks LJJ, Wong DR, de Jong DJ. Risk factors for thiopurine-induced myelosuppression and infections in inflammatory bowel disease patients with a normal TPMT genotype. Aliment Pharmacol Ther. 2017; 46(10): 953-963.
16. Bryant RV, Schultz CG, Grafton R, Hughes JC, Goess C, Schoeman M, Bartholomeusz D, Andrews JM. Mo1311 Body mass index (BMI) Is better at predicting fat mass than muscle mass in inflammatory bowel disease (IBD) patients. Gastroenterology 2013; 144: S633-S634.
17. Burchard PR, Abou Tayoun AN, Lefferts JA, Lewis LD, Tsongalis GJ, Cervinski MA. Development of a rapid clinical TPMT genotyping assay. Clin Biochem. 2014; 47(15): 126-129.
18. Carvalho AT, Esberard BC, Fróes RS, Rapozo DC, Grinman AB, Simão TA, Santos JC, Carneiro AJ, Ribeiro-Pinto LF, de Souza HS. Thiopurine-methyltransferase variants in inflammatory bowel disease: prevalence and toxicity in Brazilian patients. World J Gastroenterol. 2014; 20(12): 3327-3334.
19. Casanova MJ, Chaparro M, Molina B, Merino O, Batanero R, Dueñas-Sadornil C, Robledo P, Garcia-Albert AM, Gómez-Sánchez MB, Calvet X, Trallero MDR, Montoro M, Vázquez I, Charro M, Barragán A, Martínez-Cerezo F, Megias-Rangil I, Huguet JM, Marti-Bonmati E, Calvo

M, Campderá M, Muñoz-Vicente M, Merchante A, Ávila AD, Serrano-Aguayo P, De Francisco R, Hervías D, Bujanda L, Rodriguez GE, Castro-Laria L, Barreiro-de Acosta M, Van Domselaar M, Ramirez de la Piscina P, Santos-Fernández J, Algaba A, Torra S, Pozzati L, López-Serrano P, Arribas MDR, Rincón ML, Peláez AC, Castro E, García-Herola A, Santander C, Hernández-Alonso M, Martín-Noguerol E, Gómez-Lozano M, Monedero T, Villoria A, Figuerola A, Castaño-García A, Banales JM, Díaz-Hernández L, Argüelles-Arias F, López-Díaz J, Pérez-Martínez I, García-Talavera N, Nuevo-Siguairo OK, Riestra S, Gisbert JP. Prevalence of malnutrition and nutritional characteristics of patients with inflammatory bowel disease. J Crohns Colitis. 2017; 11(12): 1430-1439.

20. Cederholm T, Barazzoni R, Austin P, Ballmer P, Biolo G, Bischoff SC, Compher C, Correia I, Higashiguchi T, Holst M, Jensen GL. ESPEN guidelines on definitions and terminology of clinical nutrition. Clin Nutr. 2017; 36: 49-64.
21. Cederholm T, Jensen GL, Correia MITD, Gonzalez MC, Fukushima R, Higashiguchi T, Baptista G, Barazzoni R, Blaauw R, Coats A, Crivelli A, Evans DC, Gramlich L, Fuchs-Tarlovsky V, Keller H, Llido L, Malone A, Mogensen KM, Morley JE, Muscaritoli M, Nyulasi I, Pirlich M, Pisprasert V, de van der Schueren MAE, Siltharm S, Singer P, Tappenden K, Velasco N, Waitzberg D, Yamwong P, Yu J, Van Gossum A, Compher C; GLIM Core Leadership Committee; GLIM Working Group. GLIM criteria for the diagnosis of malnutrition - A consensus report from the global clinical nutrition community. Clin Nutr. 2019; 38(1) :1-9.
22. Chouchana L, Narjoz C, Roche D, Golmard JL, Pineau B, Chatellier G, Beaune P, Loriot MA. Interindividual variability in TPMT enzyme activity: 10 years of experience with thiopurine pharmacogenetics and therapeutic drug monitoring. Pharmacogenomics 2014; 15(6): 745-757.
23. Coelho T, Andreoletti G, Ashton JJ, Batra A, Afzal NA, Gao Y, Williams AP, Beattie RM, Ennis S. Genes implicated in thiopurine-induced toxicity: Comparing TPMT enzyme activity with clinical phenotype and exome data in a paediatric IBD cohort. Sci Rep. 2016; 6: 34658.
24. Coenen MJ, de Jong DJ, van Marrewijk CJ, Derijks LJ, Vermeulen SH, Wong DR, Klungel OH, Verbeek AL, Hooymans PM, Peters WH, te Morsche RH, Newman WG, Scheffer H, Guchelaar

HJ, Franke B; TOPIC Recruitment Team. Identification of patients with variants in TPMT and dose reduction reduces hematologic events during thiopurine treatment of inflammatory bowel disease. Gastroenterology 2015; 149(4): 907-917.

25. Colombel JF, Shin A, Gibson PR. AGA clinical practice update on functional gastrointestinal symptoms in patients with inflammatory bowel disease: Expert Review. Clin Gastroenterol Hepatol. 2019; 17(3): 380-390.e1.
26. Cuffari C, Dassopoulos T, Turnbough L, Thompson RE, Bayless TM. Thiopurine methyltransferase activity influences clinical response to azathioprine in inflammatory bowel disease. Clin Gastroenterol Hepatol. 2004; 2(5): 410-417.
27. Dean L. Azathioprine Therapy and TPMT Genotype. 20 Sept. 2012 [Updated 5 August 2020). In: Pratt V, McLeod H, Rubinstein W, et al. (ed.) Medical Genetics Summaries (Internet). Bethesda (MD): National Center for Biotechnology Information (US); 2012.
28. De Boer NK, Wong DR, Jharap B, de Graaf P, Hooymans PM, Mulder CJ, Rijmen F, Engels LG, van Bodegraven AA. Dose-dependent influence of 5-aminosalicylates on thiopurine metabolism. Am J Gastroenterol 2007; 102: 2747-2753.
29. Dickson AL, Daniel LL, Zanussi J, Dale Plummer W, Wei WQ, Liu G, Reese T, Anandi P, Birdwell KA, Kawai V, Cox NJ, Dupont WD, Hung AM, Feng Q, Stein CM, Chung CP. TPMT and NUDT15 variants predict discontinuation of azathioprine for myelotoxicity in patients with inflammatory disease: Real-World Clinical Results. Clin Pharmacol Ther. 2021 Sep 28.
30. Di Paolo A, Luci G. Personalized medicine of monoclonal antibodies in inflammatory bowel disease: pharmacogenetics, therapeutic drug monitoring, and beyond. Front Pharmacol. 2021; 11: 610806.
31. Domenech E, Carrion S, Garcia-Planella E, Manosa M, Gordillo J, Concepcion M, Guarner C, Cabre E. Smoking status and response to thiopurines in steroid-dependent inflammatory bowel disease. Inflamm Bowel Dis 2011; 17: 971-975.
32. Fangbin Z, Xiang G, Liang D, Hui L, Xueding W, Baili C, Huichang B, Yinglian X, Peng C, Lizi Z, Yanjun C, Feng X, Minhu C, Min H, Pinjin H. Prospective evaluation of pharmacogenomics

and metabolite measurements upon azathioprine therapy in inflammatory bowel disease: An observational study. Medicine 2016; 95: e3326.

33. Feuerstein JD, Nguyen GC, Kupfer SS, Falck-Ytter Y, Singh S. American gastroenterological association institute guideline on therapeutic drug monitoring in inflammatory bowel disease. Gastroenterology 2017; 153: 827-834.
34. Forbes A, Escher J, Hébuterne X, Kłęk S, Krznaric Z, Schneider S, Shamir R, Stardelova K, Wierdsma N, Wiskin AE, Bischoff SC. ESPEN guideline: Clinical nutrition in inflammatory bowel disease. Clin Nutr. 2017; 36(2): 321-347.
35. Frei P, Biedermann L, Nielsen OH, Rogler G. Use of thiopurines in inflammatory bowel disease. World J Gastroenterol 2013; 19(7): 1040-1048.
36. Friedman S, Blumberg RS. Inflammatory Bowel Disease. In: Jameson JL, Fauci AS, Kasper DL, Hauser SL, Longo DL, Loscalzo J, Eds. Harrison's Principles of Internal Medicine, 20th edn. New York, NY: McGraw-Hill Education; 2018.
37. Gasche C, Scholmerich J, Brynskov J, D'Haens G, Hanauer SB, Irvine EJ, Jewell DP, Rachmilewitz D, Sachar DB, Sandborn WJ, Sutherland LR. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. Inflamm Bowel Dis. 2000; 6: 8-15.
38. Gatt K , Schembri J ,Katsanos KH, Christodoulou D, Karmiris K, Kopylov U, Pontas C, Koutroubakis IE, Foteinogiannopoulou K, Fabian A, Molnar T, Zammit D, Fragaki M, Balomenos D, Zingboim N, Ben Horin S, Mantzaris GJ, Ellul P. Inflammatory bowel disease and physical activity: a study on the impact of diagnosis on the level of exercise amongst patients with IBD. Journal of Crohn's and Colitis. J Crohns Colitis. 2019; 13(6): 686-692.
39. Gennep S, Konte K, Meijer B, Heymans MW, D'Haens GR, Löwenberg M, de Boer NKH. Systematic review with meta-analysis: risk factors for thiopurine-induced leukopenia in IBD. Aliment Pharmacol Ther. 2019; 00: 1-23.
40. Gilissen LP, Bierau J, Derijks LJ, Bos LP, Hooymans PM, van Gennip A, Stockbrügger RW, Engels LG. The pharmacokinetic effect of discontinuation of mesalazine on mercaptopurine

metabolite levels in inflammatory bowel disease patients. Aliment Pharmacol Ther 2005; 22: 605–611.

41. Gisbert JP, Nino P, Rodrigo L, Cara C, Guijarro LG. Thiopurine methyltransferase (TPMT) activity and adverse effects of azathioprine in inflammatory bowel disease: long-term follow-up study of 394 patients. Am J Gastroenterol. 2006; 101: 2769-2776.
42. Ghishan FK, Kiela PR. Vitamins and minerals in inflammatory bowel disease. Gastroenterol Clin North Am. 2017; 46: 797-808.
43. Goel RM, Blaker P, Mentzer A, Fong SCM, Marinaki AM, Sandersin JD. Optimizing the use of thiopurines in inflammatory bowel disease. Ther Adv Chronic Dis. 2015; 6: 138-146.
44. Gonzalez-Lama Y and Gisbert JP. Monitoring thiopurine metabolites in inflammatory bowel disease. Frontline Gastroenterol. 2016; 7(4): 301-307.
45. Hosni-Ahmed A, Barnes JD, Wan J, Jones TS. Thiopurine methyltransferase predicts the extent of cytotoxicity and DNA damage in astroglial cells after thioguanine exposure. PLoS One. 2011; 6(12): e29163.
46. Jahnsen J. Body composition in patients with inflammatory bowel disease: a population-based study. Am J Gastroenterol. 2003; 98: 1556-1562.
47. Jakubczyk D, Leszczyńska K, Górska S. The Effectiveness of Probiotics in the Treatment of Inflammatory Bowel Disease (IBD)-A Critical Review. Nutrients 2020; 12(7): 1973.
48. Kim MJ, Choe YH. Monitoring and safety of azathioprine therapy in inflammatory bowel disease. Pediatr Gastroenterol Hepatol Nutr. 2013; 16: 65-70.
49. Kondrup J. ESPEN Guidelines for Nutrition Screening 2002. Clin Nutr 2003; 22: 415-421.
50. Kyle UG, Bosaeus I, De Lorenzo AD, Deurenberg P, Elia M, Gómez JM, Heitmann BL, Kent-Smith L, Melchior JC, Pirlich M, Scharfetter H. Bioelectrical impedance analysis. Clin Nutr. 2004; 23: 1226-1453.
51. Landi F, Camprubi-Robles M, Bear DE, Cederholm T, Malafarina V, Welch AA, Cruz-Jentoft AJ. Muscle loss: The new malnutrition challenge in clinical practice. Clin Nutr. 2019; 38: 2113-2120.
52. Lennard L. Implementation of TPMT testing. Br J Clin Pharmaco.l 2014; 77: 704-714.

53. Lewis JD, Chuai S, Nessel L, Lichtenstein GR, Aberra FN, Ellenberg JH. Use of the noninvasive components of the Mayo score to assess clinical response in ulcerative colitis. Inflamm Bowel Dis. 2008; 14(12): 1660-6.

54. Lim SL, Ong KCB, Chan YH, Loke WC, Ferguson M, Daniels L. Malnutrition and its impact on cost of hospitalization, length of stay, readmission and 3-year mortality. Clin Nutr. 2012; 31: 345-350.

55. Lim SZ, Chua EW. Revisiting the role of thiopurines in inflammatory bowel disease through pharmacogenomics and use of novel methods for therapeutic drug monitoring. Front Pharmacol. 2018; 9: 1107.

56. Liu C, Yang W, Pei D, Cheng C, Smith C, Landier W, Hageman L, Chen Y, Yang JJ, Crews KR, Kornegay N, Karol SE, Wong FL, Jeha S, Sandlund JT, Ribeiro RC, Rubnitz JE, Metzger ML, Pui CH, Evans WE, Bhatia S, Relling MV. Genome wide approach validates thiopurine methyltransferase activity is a monogenic pharmacogenomic trait. Clin Pharmacol Ther. 2016; 101: 373-381.

57. Liu YP, Wu HY, Yang X, Xu HQ, Li YC, Shi DC, Huang JF, Huang Q, Fu WL. Association between thiopurine S-methyltransferase polymorphisms and thiopurine-induced adverse drug reactions in patients with inflammatory bowel disease: a meta-analysis. PLoS One 2015; 10(3): e0121745.

58. Maaser C, Sturm A, Vavricka SR, Kucharzik T, Fiorino G, Annese V, Calabrese E, Baumgart DC, Bettenworth D, Borralho Nunes P, Burisch J, Castiglione F, Eliakim R, Ellul P, González-Lama Y, Gordon H, Halligan S, Katsanos K, Kopylov U, Kotze PG, Krustinš E, Laghi A, Limdi JK, Rieder F, Rimola J, Taylor SA, Tolan D, van Rheenen P, Verstockt B, Stoker J; ECCO-ESGAR Guideline for Diagnostic Assessment in IBD Part 1: Initial diagnosis, monitoring of known IBD, detection of complications, J Crohns Colitis 2019; 13(2): 144-164K.

59. Marinaki AM, Arenas-Hernandez M. Reducing risk in thiopurine therapy. Xenobiotica 2020; 50(1): 101-109.

60. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JC, Chan FK, Sung JJ. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet 2017; 390: 2769-2778.
61. Paine ER, Colonoscopic evaluation in ulcerative colitis, Gastroenterol Rep 2014; 2(3): 161-168.
62. Scaldaferri F, Pizzoferrato M, Lopetuso LR, Musca T, Ingravalle F, Sicignano LL, Mentella M, Miggiano G, Mele MC, Gaetani E, Graziani C, Petito V, Cammarota G, Marzetti E, Martone A, Landi F, Gasbarrini A. Nutrition and IBD: Malnutrition and/or Sarcopenia? A Practical Guide. Gastroenterol Res Pract. 2017; 2017: 8646495.
63. Raslan M, Gonzalez MC, Torrinhas RSMM, Ravacci GR, Pereira JCR, Waitzberg DL. Complementarity of Subjective Global Assessment (SGA) and Nutritional Risk Screening 2002 (NRS 2002) for predicting poor clinical outcomes in hospitalized patients. Clin Nutr. 2011; 30: 49-53.
64. Raupp D, Silva FM, Marcadenti A, Rabito EI, da Silva Fink J, Becher P, Gottschall C. Nutrition screening in public hospital emergency rooms: Malnutrition Universal Screening Tool and Nutritional Risk Screening-2002 can be applied. Public Health 2018; 165: 6-8.
65. Relling MV, Schwab M, Whirl-Carrillo M, Suarez-Kurtz G, Pui CH, Stein CM, Moyer AM, Evans WE, Klein TE, Antillon-Klussmann FG, Caudle KE, Kato M, Yeoh AEJ, Schmiegelow K, Yang JJ. Clinical Pharmacogenetics Implementation Consortium Guideline for Thiopurine Dosing Based on TPMT and NUDT15 Genotypes: 2018 Update. Clin Pharmacol Ther. 2019; 105(5): 1095-1105. doi: 10.1002/cpt.1304. Epub 2019 Jan 20. PMID: 30447069; PMCID: PMC6576267.
66. Ribaldone DG, Adriani A, Caviglia GP, Nicolò A, Agnesod D, Simiele M, Riganò D, Pellicano R, Canaparo R, Perri GD, D'Avolio A, Luzza F, Saracco GM, Astegiano M. Correlation between thiopurine S-methyltransferase genotype and adverse events in inflammatory bowel disease patients. Medicina (Kaunas) 2019; 55(8): 441.
67. Rocha R, Santana GO, Almeida N, Lyra AC. Analysis of fat and muscle mass in patients with inflammatory bowel disease during remission and active phase. Br J Nutr. 2008; 101: 676-679.

68. Roy LM, Zur RM, Uleryk E, Carew C, Ito S, Ungar WJ. Thiopurine S-methyltransferase testing for averting drug toxicity in patients receiving thiopurines: a systematic review. Pharmacogenomics 2016; 17: 633-656.
69. Shi Sum P, Asher R, Jackson R, Kneebone A, Collins P, Probert C, Dibb M, Subramanian S. Body mass index and smoking affect thioguanine nucleotide levels in inflammatory bowel disease. J Crohns Colitis. 2015; 640-646.
70. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus EV Jr, Peña AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol. 2005; 19 Suppl A: 5A-36A.
71. Skrzypczak-Zielinska M, Borun P, Bartkowiak-Kaczmarek A, Zakerska-Banaszak O, Walczak M, Dobrowolska A, Kurzawski M, Waszak M, Lipinski D, Plawski A, Slomski R. A simple method for TPMT and ITPA genotyping using multiplex HRMA for patients treated with thiopurine drugs. Mol Diagn Ther. 2016; 20: 493-499.
72. Sluiter RL, van Marrewijk C, de Jong D, Scheffer H, Guchelaar HJ, Derijks L, Wong DR, Hooymans P, Vermeulen SH, Verbeek ALM, Franke B, Van der Wilt GJ, Kievit W, Coenen MJH. Genotype-guided thiopurine dosing does not lead to additional costs in patients with inflammatory bowel disease. J Crohns Colitis. 2019; 13(7): 838-845.
73. Spekhorst LM, Visschedijk MC, Alberts R, Festen EA, van der Wouden EJ, Dijkstra G, Weersma RK; Dutch Initiative on Crohn and Colitis. Performance of the Montreal classification for inflammatory bowel diseases. World J Gastroenterol. 2014; 20(41): 15374-81.
74. Su HJ, Chiu YT, Chiu CT, Lin YC, Wang CY, Hsieh JY, Wei SC. Inflammatory bowel disease and its treatment in 2018: Global and Taiwanese status updates. J Formos Med Assoc. 2019; 118(7) :1083-1092.

75. Tripathi K, Feuerstein JD. New developments in ulcerative colitis: latest evidence on management, treatment, and maintenance. Drugs in Context 2019; 8: 212572.
76. Valentini L, Schaper L, Buning C, Hengstermann S, Koernicke T, Tillinger W, Guglielmi FW, Norman K, Buhner S, Ockenga J, Pirlich M. Malnutrition and impaired muscle strength in patients with Crohn’s disease and ulcerative colitis in remission. Nutrition 2008; 24: 694-702.
77. Van Bokhorst-de van der Schueren MA, Guaitoli PR, Jansma EP, de Vet HC. Nutrition screening tools: Does one size fit all? A systematic review of screening tools for the hospital setting. Clin Nutr. 2014; 33: 39-58.
78. Wang L, Pelleymounter L, Weinshilboum R, Johnson JA, Hebert JM, Altman RB, Klein TE. Very important pharmacogene summary: thiopurine S-methyltransferase. Pharmacogenet Genomics 2010; 20(6): 401-5.
79. Warner B, Johnston E, Arenas-Hernandez M, Marinaki A, Irving P, Sanderson J. A practical guide to thiopurine prescribing and monitoring in IBD. Frontline Gastroenterol. 2018; 9: 10-15.
80. Wędrychowicz A, Zając A, Tomasik P. Advances in nutritional therapy in inflammatory bowel diseases: Review. World J Gastroenterol. 2016; 22(3): 1045-66.
81. Wood JA, Halmos EP, Taylor KM, Gibson PR. The role of epidemiological evidence from prospective population studies in shaping dietary approaches to therapy in Crohn’s disease. Mol Nutr Food Res. 2021; 65(5) :e2000294.
82. Yarur AJ, Abreu MT, Deshpande AR, Kerman DH, Sussman DA. Therapeutic drug monitoring in patients with inflammatory bowel disease. World J Gastroenterol. 2014; 20: 3475–3484.
83. Yeshi K, Ruscher R, Hunter L, Daly NL, Loukas A, Wangchuk P. Revisiting inflammatory bowel disease: pathology, treatments, challenges and emerging therapeutics including drug leads from natural products. J Clin Med. 2020; 9(5): 1273.
84. Zhang T, Ding C, Xie T, Yang J, Dai X, Lv T, Li Y, Gu L, Wei Y, Gong J, Zhu W. Skeletal muscle depletion correlates with disease activity in ulcerative colitis and is reversed after colectomy. Clin Nutr. 2017; 36: 1586-1592.

SUPPLEMENTS

Publication I

Therapeutic Advances in Gastroenterology *Original Research*

Ther Adv Gastroenterol

2020, Vol. 13: 1–8

DOI: 10.1177/1756284820937426

© The Author(s), 2020.
Article reuse guidelines: sagepub.com/journals-permissions

Thiopurine *S*-methyltransferase genetic polymorphisms in adult patients with inflammatory bowel diseases in the Latvian population

Polina Zalizko, Juris Stefanovics, Jelizaveta Sokolovska, Natalia Paramonova, Evija Klavina, Renars Erts, Vita Rovite, Janis Klovins and Aldis Pukitis

Abstract

Background: Thiopurine methyltransferase (TPMT) plays a significant role in the metabolism of thiopurines, and, for patients with inflammatory bowel disease (IBD), it is useful to perform TPMT genotyping prior to azathioprine (AZA) treatment. In this study, we determined *TPMT* gene polymorphisms in a cohort of IBD patients in Latvia.

Methods: DNA samples were obtained from 244 IBD patients, and qPCR was performed for detection of rs1800462, rs1800460, and rs1142345 single-nucleotide polymorphisms (SNPs). Three common, non-functional *TPMT* alleles (*TPMT*2, *3B*, and **3C*) were identified (women, 51%; men, 49%). *TPMT*2, *3A, *3B*, and **3C* allelic variants detected using qPCR were consistent with restriction fragment length polymorphism (RFLP) data.

Results: Among patients, 78% had ulcerative colitis and 22% had Crohn's disease, with 93.9% of the former carrying a wild-type homozygous *TPMT*1/*1* genotype and 6.1% carrying heterozygous genotypes. The most frequent polymorphisms were *TPMT*1/*3A* (5.3%: two variants: *TPMT*3B* and *TPMT*3C*), *TPMT*1/*3C* (0.4%), and *TPMT*1/*2* (0.4%). None of the patients carried a *TPMT*3B* polymorphism and no patients were homozygous for any mutation.

Conclusion: This is the first study to identify *TPMT* gene polymorphisms in adult IBD patients in Latvia. The results indicate that the frequency of common TPMT alleles is similar to that of other European populations.

Keywords: genotyping, inflammatory bowel disease, thiopurine, thiopurine *S*-methyltransferase, *TPMT* polymorphism

Received: 18 August 2019; revised manuscript accepted: 29 May 2020.

Correspondence to:
Polina Zalizko
Faculty of Medicine, University of Latvia, Raina blv 19, Riga, 1586, Latvia
Pauls Stradins Clinical University Hospital, Riga, Latvia
zalizkopolina@gmail.com

Juris Stefanovics
Pauls Stradins Clinical University Hospital, Riga, Latvia
Faculty of Medicine, Laboratory of Personalized Medicine, University of Latvia, Riga, Latvia

Jelizaveta Sokolovska
Faculty of Medicine, Laboratory of Personalized Medicine, University of Latvia, Riga, Latvia

Natalia Paramonova
Genomics and Bioinformatics, Institute of Biology of the University of Latvia, Salaspils, Latvia

Evija Klavina
Pauls Stradins Clinical University Hospital, Riga, Latvia

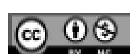
Renars Erts
Faculty of Medicine, University of Latvia, Riga, Latvia

Vita Rovite
Janis Klovins
Latvian Biomedical Research and Study Centre, Riga, Latvia

Aldis Pukitis
Pauls Stradins Clinical University Hospital, Riga, Latvia
Faculty of Medicine, University of Latvia, Riga, Latvia

Introduction

The number of patients with inflammatory bowel disease (IBD) is increasing worldwide, and, in this regard, Latvia is no exception. Azathioprine (AZA), the prodrug of mercaptopurine (MP), is used widely for the treatment of IBD.[1–3] AZA is characterized by a glucocorticoid-sparing effect, which is beneficial to patients who are unable to maintain IBD remission using glucocorticoids.[4] However, adverse effects have been recorded in approximately 10% of patients using AZA for the treatment of IBD. Among these patients, approximately 10–20% need to discontinue treatment due to these side effects.[2,5,6] Most of the adverse events occur within the first 3 months of treatment.[7] It has been observed that, at 1 and 3 months, 26% and 93%, respectively, of patients on a full dose of AZA develop complications,[8] with the most commonly reported side-effects being gastrointestinal intolerance, hepatotoxicity, infections, and bone marrow toxicity.[1,7,8] Additional complications include pancreatitis, malignancies, and allergic skin reactions.



Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).

AZA is initially metabolized in the liver *via* a non-enzymatic pathway to 6-MP. Subsequently, 6-MP is converted to its metabolites *via* an intracellular multi-enzymatic process involving three enzymes: hypoxanthine phosphoribosyl transferase (HPRT), thiopurine *S*-methyltransferase (TPMT), and xanthine oxidase (XO). The process whereby thiopurine *S*-methyltransferase is metabolized in the human body has yet to be fully established, and severe life-threatening bone marrow toxicity can result from the excess production of a drug-derived thioguanine nucleotide (TGN).[3,9,10] In terms of the therapeutic effect of AZA, it appears that 6- thioguanine (6-TG) mediates the majority of this drug's effects,[6,10,11] and accumulation of 6-TG can lead to AZA-associated adverse effects; for example, the incorporation of 6-TG into DNA can initiate delayed cytotoxicity and induce apoptotic cell death by inhibiting intracellular signalling pathways.[10] TPMT is required for the detoxification of 6-TG through *S*-methylation.[12] Thus, if the thiopurine dosage is not administered based on an individual's TPMT activity level, toxicity due to TPMT deficiency can result in treatment interruption.[13–15] In contrast, a higher than the normal TPMT activity may render patients refractory to conventional dosages of AZA.[16]

Thus, TPMT plays a significant role in the metabolism of thiopurines, with low TPMT activity being associated with altered thiopurine metabolism, overproduction of cytotoxic metabolites, and myelosuppression. In patients with IBD, *TPMT* genotyping can be performed prior to treatment to evaluate the treatment risks; however, this does not exclude that patients may or may not tolerate thiopurines. Current guidelines for thiopurine treatment mandate regular hematologic monitoring to detect leukopenia. In this study, we sought to identify *TPMT* gene polymorphisms in a cohort of IBD patients in Latvia.

Patients and methods

A total of 244 IBD patients identified from the Genome Database of the Latvian Population were included in the study after obtaining informed consent and the completion of health and heredity questionnaires, as described previously.[17] The study was performed in accordance with the Declaration of Helsinki and was approved by the Central Medical Ethics committee of Latvia (protocol no. 22.03.07/A7, no. 3/18-02-21).

From each IBD patient, we collected 20 ml of blood in an ethylenediaminetetraacetic acid (EDTA)-containing tube and 7 ml of blood in a clot-activator tube. Serum, plasma, and white blood cells were separated within 2 days of blood collection. DNA was extracted using the phenol–chloroform extraction method. Aliquots of plasma, serum, white blood cells, and DNA were stored at −80°C, avoiding cycles of re-freezing and thawing cycles.

TPMT genotypes were determined by real-time polymerase chain reaction (qPCR) using TaqMan Fluorescent Probes (TaqMan Drug Metabolism Genotyping Assays) for detection of the rs1800462, rs1800460, and rs1142345 single-nucleotide polymorphisms (SNPs). The three common non-functional *TPMT* alleles (*TPMT*2, *3B*, and **3C*) were determined. The PCR reactions amplified probes binding to DNA copies at sites of the *TPMT* gene that might contain polymorphisms and emitted fluorescent signals. All polymorphisms were analyzed using a StepOneTM Software version 2.3 Real-Time PCR System. *TPMT*2, *3A, *3B*, and **3C* allelic variants detected by qPCR were confirmed by performing PCR-restriction fragment length polymorphism (RFLP) analysis and allele-specific PCR. The results of qPCR and the alternative PCR assays were found to be entirely consistent. The DNA fragments were separated and analysed in 2.5% agarose gels and visualized by staining with ethidium bromide.

The following data were collected for each patient on the basis of responses to the health and heredity questionnaires: demographics, gender, age, nationality, and the region of Latvia where the patient was born (Vidzeme, Kurzeme, Latgale, or Zemgale). The questionnaires assessed the patient's medical history, lifestyle, and other important factors, such as smoking status, physical activity, possible risk factors of anamnesis, allergic reactions, medication intolerances, regular medications, and comorbidities.

Study participants

Our study population comprised 244 adult IBD patients, with an almost equal gender ratio (51% women and 49% men). The mean age of the participants was 43 ± 16 years. Among these patients, 78%, with a median age of 41 years (Q1−Q3 = 29.8−54.3), had ulcerative colitis,

and 22%, with a median age of 43 years (Q1−Q3 = 30.8−55.0), had Crohn's disease (p = 0.57). Women comprised 47% and 63% of the ulcerative colitis and Crohn's disease groups, respectively (Table 1).

Statistical analysis

Continuous variables are presented as the median and interquartile range (Q1−Q3) and were compared using the Mann–Whitney test. The categorical variables are expressed as the frequency and percentage and were compared using Pearson's chi-squared test with Fisher's exact test or Cramer's V effect size as appropriate. Odds ratios (ORs) are presented with 95% confidence intervals (CI). All statistical analyses were performed using SPSS Statistics version 23.0. A p-value of < 0.05 was considered statistically significant.

Results

TPMT genotyping

TPMT alleles were identified in all 244 patients, among whom we found that 93.9% were carrying a wild-type homozygous *TPMT**1/*1* genotype and 6.1% were heterozygous and harbored polymorphisms (4.9% of whom had ulcerative colitis) (Table 2). However, we found that *TPMT* polymorphisms were not consistently associated with IBD (OR: 1.15, 95% CI: 0.31−4.28, p = 0.99). The most frequent polymorphisms (5.3%) were *TPMT**1/*3A* genotype with *TPMT**3B* and *TPMT**3C* alleles. Only two patients had *TPMT**1/*3C* and *TPMT**1/*2* genotypes independently. We were unable to detect any patients carrying the *TPMT**3B* polymorphism and no patient was found to be homozygous for any mutation.

Association of TPMT polymorphisms with different clinical factors

We examined whether different clinical factors were associated with *TPMT* polymorphisms (Table 3). We accordingly observed no significant association between gender and *TPMT* polymorphisms (p = 0.21). The majority of the patients with *TPMT* polymorphisms were Caucasians and were born in Latvia outside the capital Riga. A moderate association was found for patients born in the Vidzeme region (Cramer's V = 0.2). Fisher's exact test showed a nominal statistical association between *TPMT* polymorphisms and the possible risk factors of anamnesis (working with chemicals, dust, aerosols, and lacquers, and working in chemical factories) (p = 0.04).

Table 1. Characteristics of the study group.

Diagnosis	Ulcerative colitis	*n*	190
		%	77.9%
	Crohn's disease	*n*	54
		%	22.1%
Sex	Female	*n*	124
		%	50.8%
	Male	*n*	120
		%	49.2%
Age	Median		41
	Min		17
	Max		82
Age group	Age <50	*n*	187
		%	76.6%
	Age >50	*n*	57
		%	23%
Brothers/sisters		*n*	178
		%	73%
Twin brothers/twin sisters		*n*	4
		%	0.02%

Table 2. Distribution of major TPMT alleles.

	Frequency, % of alleles	Patients, *n*
*TPMT *1/*1*	93.9	229
*TPMT *1/*3A*	5.3	13
*TPMT *1/*3C*	0.4	1
*TPMT *1/*2*	0.4	1
Total heterozygous genotypes	6.1	15

TPMT, thiopurine methyltransferase.

Table 3. Patients diagnosis and region of residence in different TPMT allele subgroups.

		TPMT genotype				
		Heterozygous		Wild type		
		n	% of total	*n*	% of total	*p value*
IBD group	Ulcerative colitis	12	4.9%	178	73.0%	
	Crohn´s disease	3	1.2%	51	20.9%	0.99
Region of Latvia	Kurzeme	2	0.9%	32	15.2%	
	Vidzeme	5	2.4%	130	61.6%	
	Latgale	1	0.5%	23	10.9%	
	Zemgale	4	1.9%	14	6.6%	0.02
City	Riga	5	2.4%	81	38.4%	
	Outside of Riga	7	3.3%	118	55.9%	0.99
Other countries	Russia	1	3.0%	13	39.4%	
	Belarus	1	3.0%	7	21.2%	
	Ukraine	0	0.0%	4	12.1%	
	Estonia	1	3.0%	0	0.0%	
	Lithuanian	0	0.0%	2	6.1%	
	Other	0	0.0%	4	12.1%	0.05
AZA	Receive	0	0.0%	22	9.0%	
	Do not receive	15	6.1%	207	84.8%	0.38

AZT, azathioprine; IBD, inflammatory bowel disease; TPMT, thiopurine methyltransferase.

Among all the included patients, 80% regularly took IBD medications and 18% had allergic reactions to antibiotics, analgesics, and other drugs. Although no statistical association was found between *TPMT* polymorphisms and drug allergy ($p=0.78$), 0.8% of all patients with positive *TPMT* polymorphisms had previously used AZA and had experienced adverse drug reactions (ADRs), such as myelosuppression and gastrointestinal intolerance. These patients had a *TPMT*1/*3A* genotype. Myelosuppression was objectively characterized as decreased white blood cells (neutropenia) and gastrointestinal intolerance was characterized as vomiting, nausea and stomach cramps. Furthermore, we found that 15% of all the patients were smokers, with a median smoking duration of 14 years (Q1–Q3 = 10.0–30.0), whereas 28% of the patients were previous smokers, with a median smoking duration of 10 years (Q1–Q3 = 4.0–20.0). However, Fisher's exact test did not reveal any statistical associations between *TPMT* polymorphisms and smoking status (OR: 1.19, 95% CI: 1.12–1.26, $p=0.14$).

Discussion

Different studies recommend that the TPMT status of patients should be determined prior to the commencement of thiopurine therapy.[1,2,10,14,18] This can be achieved by one of two methods, namely, by determining the TPMT phenotype by estimating TPMT enzyme activity in the circulating red blood cells (RBC), or through genotyping of known *TPMT* variants associated with enzyme deficiency using PCR.[14,15,18,19] The activity of the TPMT enzyme is related mainly to rs1800462, rs1800460, and rs1142345 SNPs, which are inherited co-dominantly.[10,20] In terms of AZA toxicity, large inter-individual differences are

observed due to the genetic heterogeneity of the *TPMT* gene, which has more than 40 reported allelic variants.[18,21] Nevertheless, three main patterns of TPMT enzyme activity can generally be distinguished: (a) homozygous patients with two mutant non-functional *TPMT* gene alleles and low TPMT activity, (b) heterozygous individuals with one functional and one non-functional allele and intermediate TPMT activity, and (c) homozygous wild-type (normal) individuals with two functional alleles and normal or high TPMT activity.[10,20–22]

Although known *TPMT* alleles tend to vary among different ethnic groups, four specific non-functional alleles have been identified as being more prevalent, namely, *TPMT*2*, *TPMT*3A*, *TPMT*3B*, and *TPMT*3C*. It is believed that these alleles account for between 80% and 95% of observed decreases in TPMT enzyme activity. Accordingly, these four alleles tend to be routinely targeted in most of the genotyping assays.[3,20,21] In general, the most frequently encountered allele in all populations is *TPMT*3A*, followed by *TPMT*3C*,[4,6,10,23] which is consistent with the findings of the present study.

The most common *TPMT* genotype in Caucasian populations is homozygous for the wild-type *TPMT* gene. Several studies have shown that 85–95% of patients have a wild-type genotype (93.9% in this study). In most populations, approximately 10% of individuals are heterozygotes and a further 0.3% carry homozygous variants of the non-functional *TPMT* alleles.[14,20,21,24,25] In the present study, none of the participating patients were identified as being homozygous for any mutation. *TPMT* genotyping has been demonstrated to show high sensitivity,[19,23,26] with reported sensitivities and specificities of 88.9% (81.6–97.5%) and 99.2% (98.4–99.9%), respectively. Comparatively, the approximate sensitivity and specificity of TPMT phenotyping are 91.3% (86.4–95.5%) and 92.6% (86.5–96.6%), respectively.[19]

Our study data indicated that 9% of IBD patients were undergoing thiopurine therapy at the time they were included in the study. This percentage does not include patients who have used thiopurines before and stopped, or those who did not use thiopurines at the start of the study. Indeed, most of the patients included in the study had not yet started thiopurine treatment.

With regard to the cost-effectiveness of genotype-led treatment, very recent data published by Sluiter *et al.* showed that genotype-guided thiopurine treatment in IBD patients reduces the risk of ADR among patients carrying a TPMT variant, without increasing overall healthcare costs and resulting in a quality of life comparable with that of standard treatment.[27]

The Clinical Pharmacogenetics Implementation Consortium (CPIC) has published recommendations for AZA dosing based on *TPMT* genotype. In patients with two functionally active *TPMT* alleles, the activity of the TPMT enzyme is in most cases normal or high. For these patients, the CPIC recommends the initiation of AZA therapy at normal dosage and thereafter adjusting the dose based on disease-specific guidelines. In heterozygous patients, TPMT enzyme activity is intermediate, and these patients are at an increased risk of AZA-induced myelosuppression depending on the dose. The CPIC accordingly recommends that, for heterozygous patients, AZA treatment should be initiated at 30–70% of the target dose and to titrate the dose based on tolerance. For homozygous patients with two non-functional *TPMT* alleles and consequently low TPMT enzyme activity, an alternative treatment is suggested.[1,8,21] Interestingly, in this regard, a previous study that sought to determine a safe dosage of AZA in patients with intermediate or low TPMT enzyme activity, found that *TPMT* genotyping could facilitate a reduction in adverse haematological effects by as much as 89%.[28]

Given that TMPT can vary quite extensively, depending on age, sex, ethnicity, red blood cell lifespan, red blood cell transfusion, leukemia, and treatment-related factors, these various factors should ideally be taken into consideration when interpreting the results of TPMT activity analyses.[20,22] In addition, assessments of TPMT activity may require repeated estimations, as treatment with AZA itself can induce an increase in TPMT activity. Similarly, other medications, such as 5-aminosalicylates, can reversibly inhibit TPMT activity.[29,30] Therefore, *TPMT* genotyping is considered to be a more precise and reliable method.[8] Typically, the overall concordance between *TPMT* genotype and phenotype is between 90% and 95%, although some studies have reported genotype–phenotype concordance values of approximately 60–70% in patients with low TPMT activity, whereas those for heterozygous patients are approximately 70–86%.[10,20]

However, even though the identification of *TPMT* genotype and/or phenotype can make a valuable contribution towards identifying patients with a higher risk of developing bone marrow toxicity, additional therapeutic drug monitoring is advised for those patients on AZA therapy.[11] Routine laboratory tests consist of complete blood counts (CBCs), liver chemistries, platelet counts (PC), and creatinine clearance. Some authors recommend monitoring CBC and PC at weekly intervals during the first month of AZA treatment, followed by twice monthly monitoring during the second and third months, and monthly checks thereafter. Furthermore, liver function tests should be performed at 3-monthly intervals.[19] If, however, signs of myelosuppression develop, AZA therapy should be immediately discontinued.

A recent publication by Ribaldone *et al.* describes a meta-analysis investigating the associations between *TPMT* polymorphisms and AZA-induced adverse events in patients with autoimmune diseases.[31] The results showed that *TPMT* polymorphisms were significantly associated with AZA-induced overall adverse effects, bone marrow toxicity, and gastric intolerance. However, the subgroup analysis according to ethnicity showed a significant association between *TPMT* polymorphisms and AZA-induced bone marrow toxicity in Asian populations, but not in Caucasian populations. The authors concluded that *TPMT* polymorphisms can explain a variable proportion but not all episodes of AZA-related adverse events, and, furthermore, a normal *TPMT* genotype cannot exclude the development of side effects.[31] Thus, *TPMT* genotyping before starting AZA therapy cannot replace the current practice of periodic monitoring of white blood cell count. However, this is a challenge that requires additional future research, particularly as some severe toxicities leading to life-threatening conditions remain unexplained.

It is important to note that the randomized controlled trial, TOPIC, conducted by Coenen *et al.* showed that pretreatment *TPMT* genotyping is also relevant for patients who are heterozygous for a variant in *TPMT*, not only for homozygous carriers of a genetic variant in *TPMT*.[32] The results of the TOPIC trial showed no overall effect of pretreatment *TPMT* screening followed by personalized dosing on hematologic ADRs. However, in combination with other literature, the TOPIC study shows that pretreatment *TPMT* screening followed by personalized dosing reduces the risk of leukopenia in patients carrying a genetic variant in *TPMT* and indicates that pharmacogenetic TPMT testing should be used as standard care to individualize thiopurine treatment of IBD patients.[32] Thiopurines still remain very effective in inducing and maintaining long-term remission in up to 70% of patients with IBD, and it is important to remember that patients with allelic variants should not be denied the therapeutic option of AZA, as they may tolerate this drug.[31]

Conclusion

In conclusion, our results indicate that the frequency of common TPMT alleles is similar to those of other European populations. In this study we verified the homozygous wild-type *TPMT*1/*1* genotype as the most frequently encountered genotype in ulcerative colitis and Crohn's disease patients' groups, and that *TPMT*3A* is the most prevalent polymorphism in the study population. Further, we noted the absence of both the *TPMT*3B* polymorphism and homozygous variant *TPMT* genotypes in this population. To our knowledge, this is the first study to identify *TPMT* gene polymorphisms in adult IBD patients in Latvia. We recommend that *TPMT* genotyping should be prioritized in specialized IBD centres and risk group patients for the prediction of thiopurine-induced adverse drug reactions among IBD patients, and that this genotyping should be applied with respect to personalized therapy. In the future, additional genotyping of patients experiencing adverse effects due to thiopurine treatment will be required to identify potential gene/allele–dose effects.

Acknowledgements

We wish to express our gratitude to the Genome Database of Latvian Population and the Latvian Biomedical Research and Study Centre for providing data and DNA samples.

Author contributions

We confirm that all authors have contributed to and agree on the content of the manuscript. All authors have made the following substantial contributions: PZ, JSt, VR, JK, and AP participated in the conception and design of the study. PZ, JSt, and NP performed the study and data analysis. PZ, JSt, JSo, NP, EK, RE, VR, and AP drafted the manuscript and critically revised it

for intellectual content. PZ and RE performed statistical analyses. VR participated in patient recruitment coordination and data export from databases. All authors agree to be accountable for all aspects of the work.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Polina Zalizko https://orcid.org/0000-0003-3657-6418

References

1. Benmassaoud A, Xie X, AlYafi M, *et al.* Thiopurines in the management of Crohn's disease: safety and efficacy profile in patients with normal TPMT activity-a retrospective study. *Can J Gastroenterol Hepatol* 2016; 2016: 1034834.
2. Liu YP, Wu HY, Yang X, *et al.* Association between thiopurine S-methyltransferase polymorphisms and thiopurine-induced adverse drug reactions in patients with inflammatory bowel disease: a meta-analysis. *PLoS One* 2015; 10: e0121745.
3. Skrzypczak-Zielinska M, Borun P, Bartkowiak-Kaczmarek A, *et al.* A simple method for TPMT and ITPA genotyping using multiplex HRMA for patients treated with thiopurine drugs. *Mol Diagn Ther* 2016; 20: 493–499.
4. Carvalho AT, Esberard BC, Fróes RS, *et al.* Thiopurine-methyltransferase variants in inflammatory bowel disease: prevalence and toxicity in Brazilian patients. *World J Gastroenterol* 2014; 20: 3327–3334.
5. Ardizzone S, Maconi G, Sampietro GM, *et al.* Azathioprine and mesalamine for prevention of relapse after conservative surgery for Crohn's disease. *Gastroenterology* 2004; 127: 730–740.
6. Fangbin Z, Xiang G, Liang D, *et al.* Prospective evaluation of pharmacogenomics and metabolite measurements upon azathioprine therapy in inflammatory bowel disease: an observational study. *Medicine* 2016; 95: e3326.
7. Kim MJ and Choe YH. Monitoring and safety of azathioprine therapy in inflammatory bowel disease. *Pediatr Gastroenterol Hepatol Nutr* 2013; 16: 65–70.
8. Frei P, Biedermann L, Nielsen OH, *et al.* Use of thiopurines in inflammatory bowel disease. *World J Gastroenterol* 2013; 19: 1040–1048.
9. Armstrong VW and Oellerich M. New developments in the immunosuppressive drug monitoring of cyclosporine, tacrolimus, and azathioprine. *Clin Biochem* 2001; 34: 9–16.
10. Lennard L. Implementation of TPMT testing. *Br J Clin Pharmacol* 2014; 77: 704–714.
11. Yarur AJ, Abreu MT, Deshpande AR, *et al.* Therapeutic drug monitoring in patients with inflammatory bowel disease. *World J Gastroenterol* 2014; 20: 3475–3484.
12. Burchard PR, Abou Tayoun AN, Lefferts JA, *et al.* Development of a rapid clinical TPMT genotyping assay. *Clin Biochem* 2014; 47: 126–129.
13. Gisbert JP, Nino P, Rodrigo L, *et al.* Thiopurine methyltransferase (TPMT) activity and adverse effects of azathioprine in inflammatory bowel disease: long-term follow-up study of 394 patients. *Am J Gastroenterol* 2006; 101: 2769–2776.
14. Liu C, Yang W, Pei D, *et al.* Genome wide approach validates thiopurine methyltransferase activity is a monogenic pharmacogenomic trait. *Clin Pharmacol Ther* 2016; 101: 373–381.
15. Roy LM, Zur RM, Uleryk E, *et al.* Thiopurine S-methyltransferase testing for averting drug toxicity in patients receiving thiopurines: a systematic review. *Pharmacogenomics* 2016; 17: 633–656.
16. Cuffari C, Dassopoulos T, Turnbough L, *et al.* Thiopurine methyltransferase activity influences clinical response to azathioprine in inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2004; 2: 410–417.
17. Rovite V, Wolff-Sagi Y, Zaharenko L, *et al.* Genome database of the Latvian population (LGDB): design, goals, and primary results. *J Epidemiol* 2018; 28: 353–360.
18. Coelho T, Andreoletti G, Ashton JJ, *et al.* Genes implicated in thiopurine-induced toxicity: comparing TPMT enzyme activity with clinical phenotype and exome data in a paediatric IBD cohort. *Sci Rep* 2016; 6: 34658.
19. Goel RM, Blaker P, Mentzer A, *et al.* Optimizing the use of thiopurines in inflammatory bowel disease. *Ther Adv Chronic Dis* 2015; 6: 138–146.

20. Asadov C, Aliyeva G and Mustafayeva K. Thiopurine S-Methyltransferase as a pharmacogenetic biomarker: significance of testing and review of major methods. *Cardiovasc Hematol Agents Med Chem* 2017; 15: 23–30.

21. Dean L. Azathioprine therapy and TPMT genotype. In: Pratt V, McLeod H, Rubinstein W, *et al.* (eds.) *Medical genetics summaries*. Bethesda, MD: National Center for Biotechnology Information, 2012 https://www.ncbi.nlm.nih.gov/books/NBK61999/ (2012, accessed 3 May 2016).

22. Chouchana L, Narjoz C, Roche D, *et al.* Interindividual variability in TPMT enzyme activity: 10 years of experience with thiopurine pharmacogenetics and therapeutic drug monitoring. *Pharmacogenomics* 2014; 15: 745–757.

23. Almoguera B, Vazquez L, Connolly JJ, *et al.* Imputation of TPMT defective alleles for the identification of patients with high-risk phenotypes. *Front Genet* 2014; 5: 96.

24. Broekman MMTJ, Coenen MJH, Wanten GJ, *et al.* Risk factors for thiopurine-induced myelosuppression and infections in inflammatory bowel disease patients with a normal TPMT genotype. *Aliment Pharmacol Ther* 2017; 46: 953–963.

25. González-Lama Y and Gisbert JP. Monitoring thiopurine metabolites in inflammatory bowel disease. *Frontline Gastroenterol* 2016; 7: 301–307.

26. Gennep S, Konte K, Meijer B, *et al.* Systematic review with meta-analysis: risk factors for thiopurine-induced leukopenia in IBD. *Aliment Pharmacol Ther* 2019; 50: 484–506.

27. Sluiter RL, van Marrewijk C, de Jong D, *et al.* Genotype-guided thiopurine dosing does not lead to additional costs in patients with inflammatory bowel disease. *J Crohns Colitis* 2019; 13: 838–845.

28. Feuerstein JD, Nguyen GC, Kupfer SS, *et al.* American gastroenterological association institute guideline on therapeutic drug monitoring in inflammatory bowel disease. *Gastroenterology* 2017; 153: 827–834.

29. De Boer NK, Wong DR, Jharap B, *et al.* Dose-dependent influence of 5-aminosalicylates on thiopurine metabolism. *Am J Gastroenterol* 2007; 102: 2747–2753.

30. Gilissen LP, Bierau J, Derijks LJ, *et al.* The pharmacokinetic effect of discontinuation of mesalazine on mercaptopurine metabolite levels in inflammatory bowel disease patients. *Aliment Pharmacol Ther* 2005; 22: 605–611.

31. Ribaldone DG, Adriani A, Caviglia GP, *et al.* Correlation between thiopurine S-methyltransferase genotype and adverse events in inflammatory bowel disease patients. *Medicina (Kaunas)* 2019; 55: 441.

32. Coenen MJH, de Jong, van Marrewijk CJ, *et al.* Identification of patients with variants in TPMT and dose reduction reduces hematologic events during thiopurine treatment of inflammatory bowel disease. *Gastroenterology* 2015; 149: 907–917.

Visit SAGE journals online journals.sagepub.com/home/tag

SAGE journals

Publication II

Japanese Journal of Gastroenterology and Hepatology

Review Article

Therapeutic Drug Monitoring of Thiopurine Therapy in Patients with Inflammatory Bowel Disease

Zalizko P[1,2*], Jargane I[2], Pukitis A[1,2]

[1]Pauls Stradins Clinical University, Gastroenterology, Hepatology and nutrition centre Pilsoņu Street 13, Riga, LV-1002

[2]University of Latvia, Raiņa bulvaris 19, Riga, LV-1050

Received: 15 May 2019

Accepted: 24 Jun 2019

Published: 29 Jun 2019

***Corresponding to:**

Polina Zalizko, Pauls Stradins Clinical University, Gastroenterology, Hepatology and nutrition centre, Latvia, Tel +371 26876636;E-mail:zaliz-kopolina@gmail.com

1. Abstract

The number of patients with inflammatory bowel disease (IBD) is increasing in the worldwide. Thiopurine S-methyltransferase (TPMT) plays a significant role in the metabolism of thiopurine drugs. Low TPMT activity in body is associated with pathological thiopurine drug metabolisms, overproduction of cytotoxic metabolites and myelosuppression.

The aim of this study and review was to make a comparative TPMT enzyme activity analysis using TPMT enzyme expression determination method in IBD patients who are already taking azathioprine drug therapy, with patients who have not yet begun this therapy. The long-term aim is to decrease overall expenses using azathioprine, that could be done if patients would be tested for TPMT expression level before starting therapy with azathioprine, thereby excluding this therapy for patients with higher risk of adverse side effects, reducing medical expenses treating these side effects.

20 IBD patients (55% female, n=11; 45% male, n=9) data was obtained and analysed. 70 % of patients (n=14) was diagnosed with ulcerative colitis (UC), 30 (n=6) with Crohn's disease (CD). 75% (n=15) of patients had not previously received azathioprine (Imuran 50 mg). 15% (n=3) had received azathioprine therapy, but stopped using it because of negative side effects like dyspepsia, acute pancreatitis, symptom exacerbation. 10% (n=2) was still receiving azathioprine therapy. Activity of TPMT was low (<5.5 U/mL) in 10% of patients (n=2), average (5.6-15.5 U/mL) in 5% (n=1), normal(15.6-44.0 U/mL) in 70% (n=14) and high (>44.0 U/mL) in 15% (n=3)

The results of this study and review suggests that the TPMT enzyme activity should be determined before administering azathioprine drug therapy for patients diagnosed with inflammatory bowel disease to prevent adverse reactions and evaluating treatment risks.

2. Key Words: Inflammatory bowel disease;Azathioprine;*Myelosuppression*;Thiopurine S-methyl transferase

©2019 Zalizko P. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and build upon your work non-commercially

3. Introduction

The spread of IBD patients in the world tends to increase. The treatment of these patients is an important healthcare problem nowadays, and it has been shown that effective personalized treatment reduces the risk of disability, complications and side effects. Analysing and evaluating drug metabolism plays a crucial role in predicting the effectiveness of pharmacotherapy and in preventing adverse reactions [1]. The Thiopurine drugs (azathioprine, mercaptopurine and thioguanine) are mainly used in a treatment of autoimmune diseases [2]. TPMT is one of the enzymes essential for the metabolism of thiopurines. Low TPMT enzyme activity is associated with abnormal metabolism of thiopurine drug substances, overproduction of cytotoxic metabolites, and reason of myelosuppression [3,4]. TPMT enzyme activity is regulated by genetic polymorphism. It has been found that about 0,3% of individuals are homozygous for *TPMT* mutation, while 11% have a heterozygous allele variant indicating low enzyme activity [1]. Therefore, the British National Formulary strongly recommends that the TPMT enzyme should be identified prior to starting thiopurine therapy [3].

4. Materials and Methods

All patient's blood tests were collected in the 5-7 mL vacutainer tubes. The samples were centrifuged for 30 minutes after collection for 15 minutes at 1000 rpm at 4 °C. Samples were stored frozen at -80° C, avoiding re-freezing. After collecting blood samples, a survey was completed. It includes patient of IBD diagnosis – ulcerative colitis (UC) or Cronh's disease (CD), demographic data on age, gender; as well as the duration of the disease, the history of the disease, the use of medication, intolerance and allergies, routine blood laboratory tests to assess what could affect TPMT expression. TPMT expression was determined by the ELISA using the *MyBioSurce* reagent kit Human TPMT ELISA Kit (catalogue number MBS938845).

5. Results

All 20 respondents included in the study had histological diagnosis of IBD (UC in 70%, n=14; CD in 30%, n=6).Patients had moderate to severe disease activity according Mayo score in UC patients and Crohn's Disease Activity Index (CDAI) in Crohn's disease patients. 50% of respondents (n=10) were diagnosed with particular IBD for more than 10 years ago. UC was diagnosed in 8 men and 6 women; CD was more diagnosed in 5 women and only 1 men.

Summarizing information on the usage of medications, 45% of respondents (n=9) used per oral form of mesalazine; 40% (n=8) a combination of mesalazine per oral and suppositories. 75% of respondents (n=15) have never used azathioprine before, 15% (n=3) have used it, but have stopped taking due to side effects, while 10% (n=2) used azathioprine during the study. Patients who discontinued due to adverse reactions reported side effects such as gastrointestinal symptoms and acute pancreatitis. Patient's TPMT expression ranged from 1.4 to 50 U/mL. All respondents were divided into TPMT enzyme activity: 10% (n=2) patients had low (<5.5 U/mL) TPMT activity, 5% (n=1) patient had intermediate (5.6-15.5 U/mL) activity, 70% (n=14) patients normal (15.6-44.0 U/mL) and 15% (n=3) patients high (>44.0 U/mL) TPMT activity.

6. Discussion

IBD continues to spread rapidly, it is a global health care and society problem. Patients with IBD should have early diagnostics methods and personalised treatment from the early steps of disease. As well is important therapeutic drug monitoring drug treatment, as it can decrease risks of complications and side effects and improve quality of life. All respondents in our study had an age range from 22 to 79 years, with an average age of 42 years. Both Northern Europe and USA, Canada have the highest prevalence of IBD compared to other countries. In these countries, the disease is most commonly diagnosed in patients aged 15 to 35 and the average age is 31 years [5]. In contrast, in other countries (both in Europe and Asia), the disease is most commonly diagnosed between the ages of 15 and 45 and the highest prevalence is found in young people around 20 years of age, but only 10-15% of all patients are aged 60 or over [6].

According to the respondent's data on the usage of azathioprine, most or 75% of patients have not used it, so it would be useful to find out the TPMT expression of each individual. This would make it possible to find out if the chosen therapy with one of the thiopurines will be effective and there will not be side effects. In countries such as the United States and the United Kingdom, the level of this enzyme is already established prior to initiation of therapy [2, 7].

One of the most commonly used methods is the enzymatic assay, or phenotyping, of TPMT enzyme to measure the activity of the enzyme in the blood [8]. The results of the TPMT enzyme activity test may be influenced by several factors. One of them is a recent blood transfusion that can produce false results. Medications used before may also reduce the level of this enzyme in the blood, for example if the patient has taken sulfasalazine, mesalazine, thiazide, allopurinol, salicylic acid 48 hours before the test. This is why this test is recommended to be repeated during azathioprine treatment [9].

The second approach to determining the amount of TPMT in a subject is genotyping, which determines polymorphisms in DNA. The TPMT genotypes are usually determined using the polymerase chain reaction (PCR) method. Continuing our research in the future, it would be interesting to carry out TPMT genotyping in patients with reduced TPMT enzyme activity.

Unlike phenotyping, the genotype test is not affected by external factors responsible for TPMT coding and does not need to be repeated during therapy. The sensitivity of the genotype test depends on the number of polymorphisms required to be detected [10]. Several mutation variants associated with thiopurine toxicity have been identified. The most commonly found non-functional alleles are *TPMT * 3A*, *TPMT * 3C* and *TPMT * 2* [4]. It has been shown that a patient carrying any of these TPMT alleles can accumulate large amounts of 6-TGN in the body, which may exacerbate the side effects [6].

Most patients with IBD have normal TPMT activity with two functional alleles, however, all patients receiving azathioprine therapy should be monitored and identified for TPMT enzyme activity [11]. Following the Clinical Pharmacogenetics Implementation Consortium (CPIC) for genotype and thiopurine dosing ~ 1 in 178 to 1 in 3,736 patients has a homozygous genotype with two non-functional *TPMT* alleles, which means that these patients have low / inadequate TPMT enzyme activity and have severe risk of myelosuppression during therapy. ~ 3-14% of the populations are heterozygous, with moderate risk of toxicity at 30-60% of therapy, therefore, caution and lower doses of medication are needed during therapy. In turn, 86-97% are wild-type with two functional *TPMT* alleles and high levels of enzyme activity [12].

According the increased risk of toxicity and high treatment costs, Food and Drug Administration recommends *TPMT* genotyping or phenotyping prior to initiation of thiopurine therapy. This allows patients to identify an effective starting dose of thiopurine and, if necessary, to choose other alternative medications [3,13]. CPIC has published recommendations for *TPMT* genotyping results based on the usage of azathioprine, underlining the need to consider medication substitution or a reduction in the dosage of azathioprine in patients with low or inadequate TPMT activity [2,14].

Determination of TPMT enzyme activity in IBD patients would be necessary prior to thiopurine therapy in order to prevent adverse reactions and to evaluate the risk of therapy.

References

1. Sahasranaman S, Howard D, Roy S. Clinical pharmacology and pharmacogenetics of thiopurines. European journal of clinical pharmacology. 2008;64(8): 753-767.

2. Relling M V, Gardner E E, Sandborn W J. Clinical pharmacogenetics implementation consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing: 2013 update. Clinical Pharmacology & Therapeutics. 2013;93(4): 324-325.

3. Lennard L. Implementation of TPMT testing. British journal of clinical pharmacology. 2014;77(4): 704-714.

4. Zaza G, Cheok M, Krynetskaia N. Thiopurine pathway. Pharmacogenet Genomics. 2010; 20(9).

5. Shivashankar R, Tremaine W, Harmsen S. Updated incidence and prevalence of Crohn's disease and ulcerative colitis in Olmsted County, Minnesota (1970-2010). In American Journal of Gastroenterolgy. 2014; Vol. 109: S499-S499.

6. Ha C Y. Diagnosis and Treatment of Inflammatory Bowel Disease in Older Patients. Gastroenterology &Hepatology. 2012;8(10): 669.

7. Kornbluth A, Sachar D B. Ulcerative colitis practice guidelines in adults: American college of gastroenterology, practice parameters committee. The American journal of gastroenterology. 2010;105(3): 501-523.

8. Roman M, Cabaleiro T, Ochoa D. Validation of a genotyping method for analysis of TPMT polymorphisms. ClinTher. 2012;34(4):77980878-884.

9. Ruzinov B. The impact of thiopurine-S-methyltransferase genotype on the adverse drug reactions to azathioprine in patients with inflammatory bowel diseases. BratislLekListy. 2013;114(4): 199-205.

10. Marinaki. Genetic determinants of the thiopurine methyltransferase intermediate activity phenotype in British Asians and Caucasians. Pharmacogenetics. 2003; 13(2):97-105.

11. DiPiero J, Teng K, Hicks J K. Should thiopurine methyltransferase (TPMT) activity be determined before prescribing azathioprine, mercaptopurine, or thioguanine?. Cleveland Clinic journal of medicine. 2015; 82(7): 409-413.

12. Relling MV, Gardner EE, Sandborn WJ. Clinical Pharmacogenetics Implementation Consortium Guidelines for Thiopurine Methyltransferase Genotype and Thiopurine Dosing. Clinical Pharmacology and Therapeutics. 2011 89(3): 387-391.

13. Blumenstein I, Herrmann E, Filmann N. Female patients suffering from inflammatory bowel diseases are treated less frequently with immunosuppressive medication and have a higher disease activity. Journal of Crohn's and Colitis. 2011;5(3): 203-210.

14. Mowat C, Cole A, Windsor AL. Guidelines for the management of inflammatory bowel disease in adults.

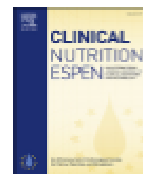
Publication III

Clinical Nutrition ESPEN 40 (2020) 193–200

Contents lists available at ScienceDirect

Clinical Nutrition ESPEN

journal homepage: http://www.clinicalnutritionespen.com



Original article

The role of body muscle mass as an indicator of activity in inflammatory bowel disease patients

Polina Zalizko [a, b, *], Tereze Hermine Roshofa [c], Laila Meija [a, c], Edgars Bodnieks [a, c], Aldis Pukitis [a, b]

[a] *Pauls Stradins Clinical University Hospital, Pilsonu Street 13, Riga, LV-1002, Latvia*
[b] *University of Latvia, Faculty of Medicine, Raina Boulevard 19, Riga, LV-1586, Latvia*
[c] *Riga Stradins University, Faculty of Medicine, Dzirciema Street 16, Riga, LV-1007, Latvia*

ARTICLE INFO

Article history:
Received 19 May 2020
Accepted 17 September 2020

Keywords:
IBD
Body muscle mass
Activity
Malnutrition
Screening tools

SUMMARY

Background & aims: Malnutrition is an objective disease activity parameter for patients with inflammatory bowel disease (IBD), particularly Crohn's Disease (CD), and is an indicator of lesion expansion or inflammatory activity. Active disease is correlated with the systemic response of the body's immune system, activating a hypermetabolic state and protein degradation *(Argiles JM, 2015)*. These conditions lead to malnutrition, which significantly increases the risk of impaired clinical outcomes, such as delayed recovery or increased mortality *(Landi F, 2019)*. Our aim was to identify malnutrition parameters associated with more pronounced metabolic status changes in IBD patients (i.e., classified as by low and high clinical activity) as an indicator of disease activity.

Methods: This prospective pilot study included hospitalised patients aged ≥18 years, with an established diagnosis of IBD, with no medical history of surgical interventions. IBD patients were divided into those with low clinical activity indexes (CD activity index [CDAI] <150 for CD and Mayo <4 for ulcerative colitis [UC]) and those with high clinical activity indexes (CDAI >150 for CD and Mayo >4 for UC). Patients were assessed twice using the Nutritional Risk Score (NRS2002) and Malnutrition Universal Screening Tool (MUST) and 48 body bioelectrical impedance analysis (BIA) measurements were taken. A control group consisting of heathy age- and sex-matched individuals was used for comparison.

Results: Fifty hospitalised patients (median age, 36.5 IQR: 28.5–51.5 years) were enrolled, of which 44% (n = 21) were female and 56% (n = 27) were male. Of these, 48% (n = 23) patients were diagnosed with CD and 52% (n = 25) with UC. The median CDAI was 128 (IQR = 6.0–207.0) and Mayo score was >4 (IQR = 1.0–8.0). The study group comprised 48% (n = 23) patients with low IBD activity and 52% (n = 25) of patients with high IBD activity. According to the NRS2002, 31% (n = 15) patients were nutritionally at risk and in need of nutritional support and an additional 24% (n = 12) had low-risk requiring observation, without necessity for additional nutritional care. According to the MUST score, 40% (n = 19) of patients had a high-risk of malnutrition requiring a nutritional care plan and 19% (n = 9) were of low-risk. Overall, 31% (n = 17) of patients received enteral oral feeding and 10% (n = 4) required additional parenteral feeding. The group with low IBD activity showed a considerably lower score on both screening tools (NRS2002 $p = 0.007$; MUST $p < 0.001$). Comparing BIA results between IBD patients and the control group, the median BMI was lower for the CD (21.10 [IQR = 19.2–23.3]) than for the control group (23.4 [IQR = 21.5–25.8]) ($p = 0.014$). In addition, visceral fat mass was lower in CD (−4,00 [IQR = −12.1 to 5.6]) than in the control group (7.85 [IQR = −0.9-18.2]) ($p = 0.003$). In terms of deviation from standard weight, 39% (n = 9) of CD patients showed reduced %body fat, while this was observed in only 19% (n = 5) of UC patients. Reduced muscle mass was observed in 48% (n = 11) of CD patients and in 19% (n = 4) of UC patients, while only 13% (n = 6) of all IBD patients had reduced BMI.

Conclusions: IBD patients with high disease activity indices had a noticeably increased risk for malnutrition (according to NRS2002 scores), taking into consideration not only IBD activity, but also increased weight loss and loss of appetite. Most CD patients in both the low and high disease activity groups had

* Corresponding author. Pauls Stradins Clinical University Hospital, Pilsonu Street 13, Riga, LV-1002, Latvia. Fax: +371 67095387.
E-mail address: polina.zalizko@stradini.lv (P. Zalizko).

https://doi.org/10.1016/j.clnesp.2020.09.023
2405-4577/© 2020 European Society for Clinical Nutrition and Metabolism. Published by Elsevier Ltd. All rights reserved.

reduction in muscle mass, which was not evaluated in UC patients. Identification of the reduction in soft lean muscle mass in CD patients can be used as an anticipatory indicator of disease activity.
© 2020 European Society for Clinical Nutrition and Metabolism. Published by Elsevier Ltd. All rights reserved.

Abbreviations

BIA	bioelectrical impedance analysis
BMI	body mass index
CD	Crohn's Disease
CDAI	Crohn's Disease activity index
CRP	C-reactive protein
F	female
FFM	fat-free mass
HGB	haemoglobin
HTC	haematocrit
IBD	inflammatory bowel disease
IQR	interquartile range
M	male
MBF	metabolic body fat
MUST	Malnutrition Universal Screening Tool
NRS2002	Nutritional Risk Score
PBF	percent body fat
RBC	red blood cells
SGA	subjective global assessment
SLM	soft lean mass
TBW	total body water
UC	ulcerative colitis
WBC	white blood cells
WHR	waist-to-hip circumference ratios

1. Introduction

Active inflammatory bowel disease (IBD) is correlated with the systemic response of the body's immune system, activating a hypermetabolic state and protein degradation [1]. These conditions lead to malnutrition, which significantly increases the risk of impaired clinical outcomes, such as delayed recovery or increased mortality [2,3].

Currently IBD has become a global disease, with increasing incidence worldwide [4]. The widespread involvement of gastrointestinal tract disorders raises particular attention to nutritional requirements of IBD patients [3]. Several factors can affect nutritional status and promote the development of malnutrition, such as the duration and activity of disease. Other components that influence development of malnutrition include increased energy requirements, reduced nutritional uptake, reduced break down and absorption of nutrients, and malabsorption [5].

Malnutrition is associated with negative clinical outcomes and higher rates of IBD mortality [6]. Malnourished patients are more likely to undergo repeated admissions within 15-day periods and higher mortality rates in three-years' time. Patients are at increased risk of complications, which therefore increases length of hospital stays and treatment costs [7]. Screening in Emergency Units has shown a prevalence of nutritional risk of 35.3% and 28.5% according to the screening tools Nutritional Risk Screening Score 2002 (NRS2002) and Malnutrition Universal Screening Tool (MUST), respectively. Hence, this raises importance of detecting undernutrition as early as possible [8].

Several screening tools are recommended by the European Society of Clinical Nutrition and Metabolism, including the NRS2002 the MUST for nutritional assessment in hospital settings [9]. As reported by review of 83 studies both screening tool performances were rated fair to well for predicting clinical outcomes in adult patients [10]. The identification of new metabolic markers, sensitive for early diagnosis of IBD-induced malnutrition, will be a future challenge of targeted IBD care.

Our aim of this study was to identify malnutrition parameters associated with more pronounced metabolic status changes in IBD patients (i.e. classified as by low and high clinical activity) as an anticipatory indicator of disease activity.

2. Materials and methods

2.1. Patients

The ESPEN practical guideline: Clinical Nutrition in inflammatory bowel disease, 2019 emphasizes that for adult IBD patients, the risk of malnutrition may be assessed using validated screening tools, including both NRS2002 and MUST [5]. Fifty hospitalised patients were screened using both the NRS2002 and MUST scores [9]. Both tools were developed to assess the nutritional status of patients and to predict possible outcomes associated with nutritional status. The MUST assesses three factors, such as the body mass index (BMI), and weight loss in the past 3–6 months and the severity of illness. The NRS2002 includes the same questions but additionally evaluates nutritional intake over the week prior to assessment and adds an additional point for elderly patients (i.e. aged >75 years). NRS2002 also stratifies diseased patients according to severity of disease [5,9]. Patients were screened twice if their scores indicated a nutritional risk on the first assessment. None of the patients had any autoimmune diseases or surgical interventions in the anamnesis. IBD patients were divided into two groups with low clinical activity indexes (CDAI < 150 for CD and Mayo <4 for UC) and high clinical activity indexes (CDAI > 150 for CD and Mayo >4 for UC), and were further divided into smaller groups of UC and CD separately (Fig. 1). The study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics committee.

2.2. Bioelectrical impedance analysis

Bioelectrical impedance analysis (BIA) was performed for 48 patients at admission day. BIA was not carried out for two patients, because both were bedridden due to severe exacerbation of their underlying illness. The *GENIUS 2002 (Jawon Medical, Korea)* was used to assess BIA, and measurements were performed at least 3 h after eating, as this may reduce small errors in impedance determination [11]. The instrument requires patient to be in the standing position and eight touch electrodes with frequency range 5, 50, 250 kHz are used to take measurements. Electrical current penetrates tissues in various frequencies based on their different

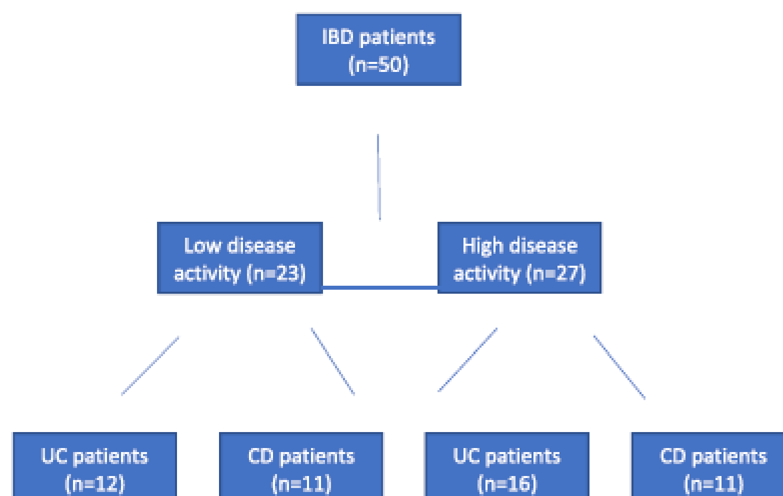


Fig. 1. Diagram of study flow. IBD, inflammatory bowel disease; UC, ulcerative colitis, Crohn's disease.

electrical features and depending on hydration or nutritional status; multifrequency BIA increases the accuracy of measurements [12]. The whole body was measured, by subdividing it into various segments. Furthermore, weight (kg), BMI, fat-free mass (FFM) (kg), soft lean mass (SLM) (kg), metabolic body fat (MBF) (kg), total body water (TBW) (kg), basal metabolic rate (kcal), total energy expenditure (kcal), visceral fat (%) were assessed.

2.3. Control group

We enrolled 58 individuals from the general population as the control group. BIA was performed if person met the inclusion criteria: age ≥ 18 years old and age-matched to the patient group, with no food intake ≥3 h prior to testing, and with no nutritional risks identified using both screening tools (NRS2002 and MUST), which assured that the control group did not harbour any active disease that would interfere with the study results. The control group comprised 48 individuals; the selection ensured appropriate age- and sex-matched (i.e. with equal male to female ratio) controls for the study group.

2.4. Statistical analysis

For the statistical analysis, we used *SPSS version 22 (IMDB Statistical Package for the Social Sciences 22)*. Statistically significance was set at p-values ≤0.05. Data are indicated as median (interquartile range [IQR] 25th-75th percentile) or mean ± SD for normally distributed data sets. The independent sample Kruskal–Wallis test was used to identify significant statistical differences between groups of independent variables, for non-parametric datasets, and the one-way ANOVA was used for data with normal distribution. After a non-paired analysis was carried out, the Mann-Whitney U test was performed to evaluate variance between pair-matched groups. For related samples, the Wilcoxon test was used to compare two series of scores in the same group.

3. Results

3.1. Descriptive statistics

Among the 48 IBD patients included in the analysis, 52% (n = 25) were UC patients and 48% (n = 23) were CD patients. Disease activity was measured by the Mayo score for UC patients with a median score of 4 (IQR: 1.0–6.25) and CDAI was used for CD patients with median result of 128 (IQR: 56.0–207.0). Of the IBD patients, those with a low activity CDAI score of <150 for CD or Mayo<4 for UC were 48% (n = 23), and high activity CDAI > 150 for CD or Mayo>4 for UC comprised 52% (n = 25) of patients. For IBD patients, the median age of the patient IBD group was 1 year younger at 35.5 years (IQR: 27.5–49.8) of those assessed by BIA. The median age for the control group was 32.0 years (IQR: 26.0–41.8), although there was a noticeable age gap compared to patient group, the pair-matched analysis using the Kruskal–Wallis test, did not show any statistically significant difference between these two groups (p = 0.198). Characteristics of the study group are shown in Table 1.

Table 2 shows the assessment of general laboratory variables. Patients had several micronutrient deficits, but no statistical relationship with the screening tools was identified. Nonetheless, patients who appeared to be in clinical remission and with no signs of undernutrition could still harboured micronutrient deficits.

3.2. Nutritional screening

Of the patients screened, 31% (n = 15) revealed to be in a high-risk group, 25% (n = 12) had medium-risk, but 44% (n = 21) had low nutritional risk according to the NRS2002. The MUST score revealed nearly inversely proportional values in the high- and medium-risk group: 40% (n = 19) of patients had a high-risk score, while 19% (n = 9) had a medium-risk of malnutrition (Table 3).

Despite these differences in high- and medium-risk groups, we observed a strong positive correlation between both screening tools NRS2002 and MUST (Spearman's correlation coefficient, rho = 0.85; p < 0.001) (Fig. 2).

Previous studies have reported that disease activity significantly affects nutritional status of patients. An increase in the activity index, increases the risk of malnutrition. We observed a moderate positive correlation between NRS2002 results and disease activity index (Spearman's correlation coefficient, rho = 0.577; p < 0.001), but a weakly positive correlation was observed using MUST scores (Spearman's correlation coefficient, rho = 0.429; p < 0.001) (Table 4). We further evaluated whether there were significant differences between the risk of undernutrition and disease activity.

Table 1
Description of the study group.

		Patients (n = 50)	Percent
Diagnosis	UC	25	52%
	CD	23	48%
Clinical activity	Asymptomatic	21	44%
	Mild	15	31%
	Moderate	11	23%
	Severe to Fulminant	1	2%
Sex	Female	19	40%
	Male	29	60%
Smoker	Yes	8	17%
	No	40	83%
Alcohol consumption	No	23	48%
	Once a week	6	13%
	Once a month	13	27%
	Less than once a month	6	13%

CD, Crohn's Disease; UC, ulcerative colitis.

Table 2
Comparison of laboratory tests results of the low and high IBD activity group.

Parameter	Low activity group		High activity group	
	Patients (n = 23)	Percent %	Patients (n = 25)	Percent %
CRP (>5 mg/l)	4	17.4	19	76%
Albumin (<35 g/l)	6	26.1	5	20%
RBC (Male<4.5 × 10^9/l; Female<4.2 × 10^9/l)	9	39.1	14	56%
HTC (<40%)	14	60.9	15	60%
HGB (M < 130 g/l; F < 120 g/l)	13	56.5	10	40%
WBC count (>10 × 10^{12}/l)	4	17.4	5	20%
Platelet count (>400 × 10^9/l)	5	21.7	5	20%
Creatinine (<62 μmol/l)	8	34.8	7	28%
Creatinine (>115 μmol/l)	1	4.3	1	4%
Glucose (>6 mmol/l)	1	4.3	2	8%
Ferritin (<22 ng/ml)	8	34.8	7	28%

CRP, C-reactive protein; RBC, red blood cells; M, male; F, female; HTC, haematocrit; HGB, haemoglobin; WBC, white blood cells.

Table 3
Comparison of risk groups according to the NRS2002 and MUST scores.

Risk group	NRS2002	MUST
High-risk	31% (n = 15)	40% (n = 19)
Medium-risk	25% (n = 12)	19% (n = 9)
Low-risk	44% (n = 21)	42% (n = 20)

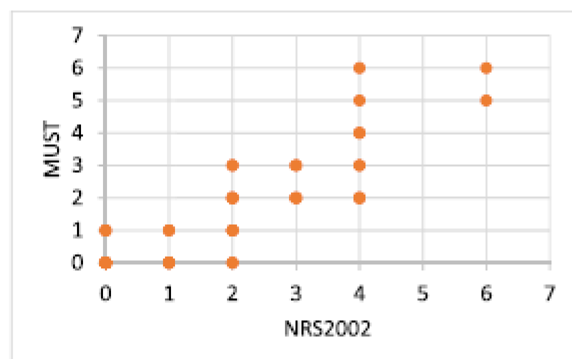


Fig. 2. Spearman's correlation of MUST and NRS2002. NRS2002, Nutritional Risk Score 2002; MUST, Malnutrition Universal Screening Tool.

A pair-matched analysis revealed a significant difference, among patients in clinical remission and patients with average scores (NRS2002 p = 0.001; MUST p = 0.026, Kruskal–Wallis test) or high disease activity (NRS2002 p = 0.023; MUST p = 0.038, Kruskal–Wallis test). In Fig. 3, extreme values are shown, which indicate that patients were in clinical remission, but were still at risk of undernutrition. This reveals the importance of regular screening, even though disease activity might not be high. Differences in scores might be due to gradual weight loss, since the MUST evaluates weight changes over a 3-6-month period.

Patients at risk for malnutrition were evaluated twice using both screening tools (NRS2002 and MUST) to estimate reduction in undernutrition risk, after receiving clinical feeding. Statistical analysis showed a reduction in score measured by the screening tools (related sample Wilcoxon test, p = 0.020). We should take in consideration that patients also received treatment that reduced disease activity therefore also reducing points scored using the screening tools.

Clinical nutrition was administered to 18 patients; enteral oral feeding was prescribed to 17 of these patients. Additionally, four patients received parenteral peripheral feeding and one patient was switched to central venous feeding.

The NRS2002 was used to screen 17 patients in the high-risk group; 88% (n = 15) received clinical feeding and 12% (n = 2) did not. A slightly lower percentage, 86% (n = 18) of patients received feeding of the 21 patients who were in high-risk group based on the MUST score. Additional information on nutritional status of the groups is presented in Table 5.

3.3. Bioelectrical impedance analysis

Figure 4 reveals normal BMI values were present for most patients, although an imbalanced body composition with changes in

Table 4
Spearman's correlation of activity index: NRS2002 vs MUST.

		NRS2002	MUST
Activity index	*rho*	0.577	0.429
	p-value	<0.001	<0.001
NRS2002	*rho*		0.830
	p-value		<0.001

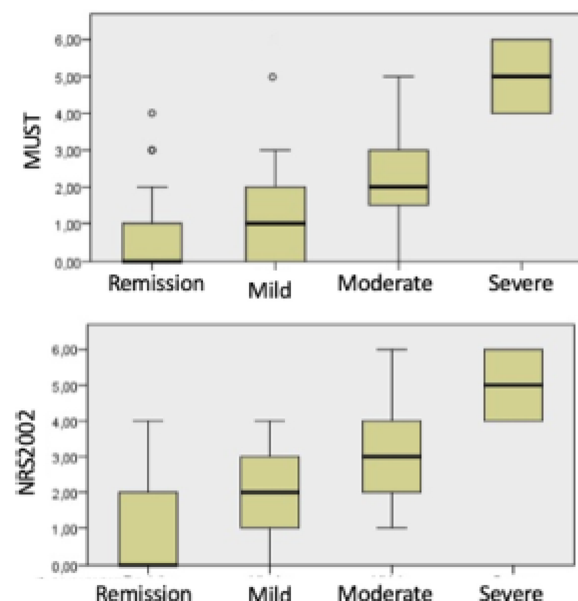


Fig. 3. Screening tool results according to disease activity. Kruskal–Wallis test results displayed in box chart. NRS2002, Nutritional Risk Score 2002; MUST, Malnutrition Universal Screening Tool.

percent body fat (PBF) and SLM was observed in a large proportion of patients. This indicated that even though patients have BMI within normal values, there is a high chance of having imbalance in body composition. Therefore, the evaluation of lean mass and PBF is important.

Figure 5 demonstrates the changes in muscle mass from normal values. Most CD patients presented a reduction in muscle mass not only in those patients with high disease activity, but also in patients with moderate and low disease, which contrasted with the greater part of UC patients that had normal or increased muscle mass. CD patients are more prone to reduction in muscle mass when disease activity is high. Only few individuals of the control group (8% [n = 4]) had muscle mass under the normal values.

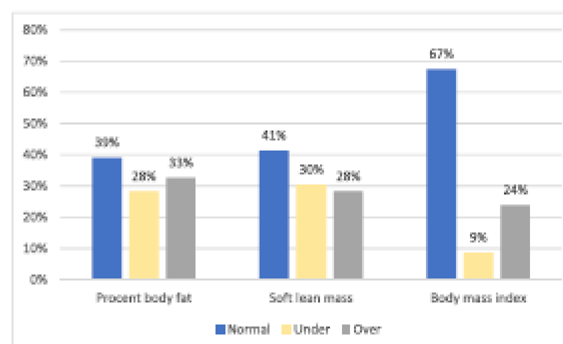


Fig. 4. Differences in BIA among patient groups. BIA, bioelectrical impedance analysis.

To assess the relationship between screening tools and BIA, a correlation analysis was used. In Table 6 the correlation of BIA values and the results assessed by MUST and NRS2002 is shown. A weak negative correspondence between BIA results (including patient weight, BMI, and %visceral fat) and the MUST score was found. The NRS2002 Screening tool did not show any significant relationship with BIA analysis ($p > 0.50$) (see Table 7).

Data obtained by BIA was compared to those of the population control group. Statistically important differences were observed in BMI, %visceral fat, body fat (kg), and hip-waist ratio. Other values also seemed to vary, but analysis did not show statistically significant differences.

Analysis of body composition demonstrated considerably lower BMI values ($p = 0.014$) and %visceral fat mass ($p = 0.003$) in CD patients than in controls. Patients with UC did not show any statistically significant reduction of values when compared to the control group. In contrast, patients with UC showed higher FM in kg ($p = 0.046$) and increased waist-hip ratio ($p = 0.011$).

High and low disease activity showed significant differences across nutritional screening scales (Table 8). Patients having a high activity index have a noticeably increased risk for malnutrition, taking into consideration not only disease activity, but also increased weight loss and loss of appetite.

Table 5
Additional nutrition.

Nutritional supplement	Contains one unit	Patients received
Nutridrink	2.4 kcal/ml, 125 ml (bottle); Fat: 11.6 g OGH: 37.1 g Protein: 12 g	- 57% (n = 8) 4 bottles/day - 28% (n = 4) 3 bottles/day - 14.2% (n = 2) 2 bottles/day
Cubitan	1.25 kcal/ml, 200 ml (bottle) Fat: 7.0 g OGH: 29 g Protein: 17.6 g	- (n = 1) 3 bottles/day - (n = 2) 2 bottles/day
Protifar	8 kcal, 1 spoon Fat 1.6 g OGH, <1.6 g	- 50% (n = 9) 3 spoons/day
Kabiven	1448 ml, 1000 kcal, Amino acids: 456 ml, Dextrose 788 ml 13% Lipids 204 ml	- 22.2% (n = 4)

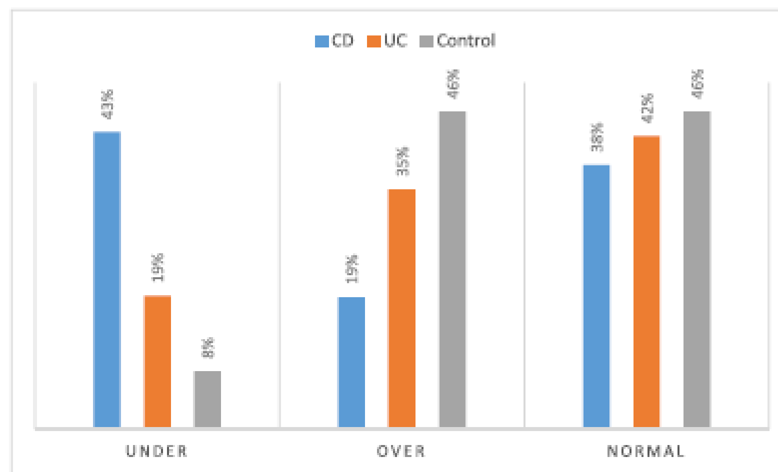


Fig. 5. Differences in muscle mass in among patients in the sample. UC, ulcerative colitis, Crohn's disease.

Table 6
Comparison of BIA values and malnutrition screening scores (NRS2002, MUST).

Scale	BIA							
	Weight		SLM		BMI		Visceral fat	
	rho	p	rho	p	rho	p	rho	p
NRS2002	−0.133	>0.050	−0.083	>0.050	−0.184	>0.050	−0.198	>0.050
MUST	−0.305	0.003	−0.224	0.019	−0.329	<0.001	−0.351	<0.001

Spearman's correlation coefficient was used for the analysis.
BIA, bioelectrical impedance analysis; BMI, body mass index; SLM, soft lean mass.

Table 7
Comparison of parameters between the study groups and the control group.

	UC	CD	Control	p-value
One-way ANOVA				
	Mean ± SD			
Weight	75.8 ± 15.1	68.7 ± 14.8	73.5 ± 13.9	0.232
PBF (%)	24.1 ± 9.9	21.3 ± 7.5	21.8 ± 9.9	0.355
Muscle mass deviation from normal	1.58 ± 4.4	0.6 ± 3.1	2.9 ± 4.6	0.143
Fat deviation from normal	4.8 ± 9.2	1.3 ± 6.8	1.8 ± 5.0	0.122
Kruskal–Wallis test				
	Median [IQR]			
Muscle mass	53.2 [44.0–60.8]	49.6 [41.1–57.3]	55.8 [42.9–61.4]	0.425
Proteins	41.7 [34.4–47.2]	38.7 [32.2–44.4]	12.5 [9.6–13.8]	< 0.001
Minerals	4.30 [3.8–5.0]	3.8 [3.4–4.7]	4.3 [3.6–4.7]	0.212
TBW	41.7 [34.4–47.2]	38.7 [32.20–44.35]	43.1 [33.4–47.4]	0.454
Basal metabolism	1469.5 [1240.8–1643.0]	1503.0 [1196.00–1607.0]	1518.5 [1266.5–1711.8]	0.407
Weight deviation from normal	8.1 [-1.1-19.7]	4.1 [-8.4-6.3]	4.2 [-1.2-12.2]	0.102
Mann-Witney U test, pair-matched analysis				
	Median [IQR]			
WHR	0.9 [0.8–0.9]		0.8 [0.7–0.8]	0.011
TBF (kg)	17.8 [12.3–25.9]		14.5 [11.2–18.6]	0.046
BMI		23.4 [19.2–23.3]	21.1 [21.5–25.8]	0.041
Visceral fat %		−4.0 [-12.1-5.6]	7.9 [-0.9-18.2]	0.014

UC, ulcerative colitis; CD, Crohn's Disease; PBF, percent body fat; TBW, total body water; WHR, waist-to-hip circumference ratios; TBF, total body fat; BMI, body mass index.

4. Discussion

Malnutrition can be subdivided into several subtypes, depending on the causative factor, either disease or a lack of necessary nutrition. Due to many possible causes, there is a wide definition of malnutrition and undernutrition making it complicated to

Table 8
Comparison of high and low disease activity groups.

Score	High activity	Low activity	*P value*
NRS2002	0.30 [2.00–4.00]	0.00 [0.00–2.00]	0.007
MUST	2.00 [1.00–3.00]	0.00 [0.00–1.00]	<0.001

determine a diagnosis [13]. Since it is important to establish an accurate nutritional status for patients, numerous nutritional screening tools have been developed; however, none is considered the gold standard for nutritional assessment [14]. Both screening tools NRS2002 and MUST use in this study are recommended by EPSEN guidelines [9]. Our data indicated strong agreement between both scales on evaluation of the nutritional status of the patient. Similar agreement was reported by Raupp et al. [8]. Nutritional assessment was performed 48 h after admission to hospital using the NRS2002 and MUST. Both results were compared to a subjective global assessment (SGA) and they presented a good agreement in nutritional status evaluation [8]. However, it has been pointed out that NRS2002 is more specific, due to scaling of disease activity, while MUST establishes patients with severe disease by defining them as "high nutritional risk"; therefore, it may overestimate nutritional risk. Another possible reason for higher scores obtained by MUST, is that this scale evaluates patients over a longer period. Thus, if weight loss is gradual, and for example the patient showed a weight loss of over less than 5% over the previous 3 months, no points are given by the NRS2002. In our study, there were two extreme values where patients lost weight over a longer period, hence receiving points by the MUST scale, but not by the NRS2002.

A previous study by Valentini et al. evaluated the nutritional status in IBD patients [15]. Their results showed that patients in remission who seemed well-nourished by screening tools, tended to have reduced body cell mass, reduced hand grip strength, and micronutrient deficit. These results indicated that nutritional deficit occurs in same ratio for UC and CD patients, in contrast to our data which showed that CD patients were slightly more affected [15]. Several studies are in agreement with the present findings, whereby CD patients are in higher nutritional risk than UC patients, even in clinical remission [14,16,17]. This might be explained by the involvement of the small bowel, which leads to impaired absorptive function and loss of nutrients due to fistulas [18]. Chronic inflammatory processes and lack of physical activity stimulate muscle deterioration, leading to sarcopenia [1,13]. In our study, patients had several micronutrient deficits, but no statistical relationship with screening tools was found. Patients who appear to be in clinical remission and without signs of undernutrition may still have micronutrient deficit.

Patients with UC show better body composition parameters than CD patients. Sarcopenia in UC patients is more dependent on disease activity, where change in muscle mass correlates with an increase in the Mayo score. Patients in remission show better body composition values, additionally for UC patients after colectomy values have been shown improve [19].

Most patients had a BMI within normal values, while in many cases a disproportion of body composition was observed. The later raises the need for a closer investigation of the patient's nutritional status than merely assessment of the BMI. It has been reported that BMI has a better correlation with the Fat Mass Index, therefore BMI is better at predicting body fat mass than muscle mass [20].

Not all patients screened to be at nutritional risk received clinical feeding. This was determined at the physician's discretion, since some of patients were able to eat nutritionally rich food and disease activity was controlled adequately with medical therapy, thus they were not given additional feeding. Patients who received nutritional support without being in high-risk group, were previously identified as "nutritionally at risk"; thus, they continued to receive nutritional support as a part of treatment. Nutritional management should be defined depending on the patient's nutritional status, and considering necessary requirements of energy and nutrients, appropriate route of administration to adequately set goals and duration of treatment to achieve them [13].

The BIA showed a statistical correspondence with the MUST scale. More significant changes in body composition were observed in CD patients. Among diseased patients with normal BMIs, an imbalance in body composition can be observed, stressing the importance of broader body composition analysis.

In conclusion IBD patients with a high disease activity index had a noticeably increased risk for malnutrition (according to the NRS2002 scale), considering not only IBD activity, but also increased weight loss and loss of appetite. Most CD patients showed a reduction in muscle mass in both groups with low and high disease activity, which was not evaluated in UC patients. Identification of the reduction in muscle mass (*soft lean muscle mass*) in CD patients can be used as an anticipatory indicator of the disease activity.

Statement of authorship

Aldis Pukitis and Polina Zalizko contributed in conception and design of the research. Tereze Hermine Roshofa and Polina Zalizko contributed in search, data collection, extraction, and analysis. All authors contributed to the drafting and review of the manuscript, discussion, and revision. All authors approved the final manuscript.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no conflict of interest. No author received financial assistance to carry out this research.

Acknowledgements

The English text of this paper has been revised by professional editors at Editage, a division of Cactus Communications, in cooperation with Taylor & Francis Group.

References

[1] Argilés JM, Busquets S, Stemmler B, López-Soriano FJ. Cachexia and sarcopenia: mechanisms and potential targets for intervention. Curr Opin Pharmacol 2015;22:100–6.

[2] Landi F, Camprubi-Robles M, Bear DE, Cederholm T, Malafarina V, Welch AA, et al. Muscle loss: the new malnutrition challenge in clinical practice. Clin Nutr 2019;38:2113–20.

[3] Friedman S, Blumberg RS. Inflammatory bowel disease. In: Jameson JL, Fauci AS, Kasper DL, Hauser SL, Longo DL, Loscalzo J, editors. Harrison's principles of internal medicine. 20th ed. New York, NY: McGraw-Hill Education; 2018.

[4] Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet 2017;390:2769–78.

[5] Bischoff SC, Escher J, Hébuterne X, Kłęk S, Krznaric Z, Schneider S, et al. ESPEN practical guideline: clinical nutrition in inflammatory bowel disease. Clin Nutr 2020;39:632–53.

[6] Raslan M, Gonzalez MC, Torrinhas RSMM, Ravacci GR, Pereira JCR, Waitzberg DL. Complementarity of subjective global assessment (SGA) and nutritional risk screening 2002 (NRS 2002) for predicting poor clinical outcomes in hospitalized patients. Clin Nutr 2011;30:49–53.

[7] Lim SL, Ong KCB, Chan YH, Loke WC, Ferguson M, Daniels L. Malnutrition and its impact on cost of hospitalization, length of stay, readmission and 3-year mortality. Clin Nutr 2012;31:345–50.

[8] Raupp D, Silva FM, Marcadenti A, Rabito EI, da Silva Fink J, Becher P, et al. Nutrition screening in public hospital emergency rooms: malnutrition Universal Screening Tool and Nutritional Risk Screening-2002 can be applied. Publ Health 2018;165:6–8.

[9] Kondrup J. ESPEN guidelines for nutrition screening 2002. Clin Nutr 2003;22:415–21.

[10] Van Bokhorst-de van der Schueren MA, Guaitoli PR, Jansma EP, de Vet HC. Nutrition screening tools: does one size fit all? A systematic review of screening tools for the hospital setting. Clin Nutr 2014;33:39–58.
[11] Kyle UG, Bosaeus I, De Lorenzo AD, Deurenberg P, Elia M, Gómez JM, et al. Bioelectrical impedance analysis—part II: utilization in clinical practice. Clin Nutr 2004;23:1430–53.
[12] Kyle UG, Bosaeus I, De Lorenzo AD, Deurenberg P, Elia M, Gómez JM, et al. Bioelectrical impedance analysis—Part I: review of principles and methods. Clin Nutr 2004;23:1226–43.
[13] Cederholm T, Barazzoni R, Austin P, Ballmer P, Biolo G, Bischoff SC, et al. ESPEN guidelines on definitions and terminology of clinical nutrition. Clin Nutr 2017;36:49–64.
[14] Ghishan FK, Kiela PR. Vitamins and minerals in inflammatory bowel disease. Gastroenterol Clin N Am 2017;46:797–808.
[15] Valentini L, Schaper L, Buning C, Hengstermann S, Koernicke T, Tillinger W, et al. Malnutrition and impaired muscle strength in patients with Crohn's disease and ulcerative colitis in remission. Nutrition 2008;24:694–702.
[16] Jahnsen J. Body composition in patients with inflammatory bowel disease: a population-based study. Am J Gastroenterol 2003;98:1556–62.
[17] Ananthakrishnan AN, Khalili H, Higuchi LM, Bao Y, Korzenik JR, Giovannucci EL, et al. Higher predicted vitamin D status is associated with reduced risk of Crohn's disease. Gastroenterology 2012;142:482–9.
[18] Rocha R, Santana GO, Almeida N, Lyra AC. Analysis of fat and muscle mass in patients with inflammatory bowel disease during remission and active phase. Br J Nutr 2008;101:676–9.
[19] Zhang T, Ding C, Xie T, Yang J, Dai X, Lv T, et al. Skeletal muscle depletion correlates with disease activity in ulcerative colitis and is reversed after colectomy. Clin Nutr 2017;36:1586–92.
[20] Bryant RV, Schultz CG, Grafton R, Hughes JC, Goess C, Schoeman M, et al. Mo1311 Body mass index (BMI) Is better at predicting fat mass than muscle mass in inflammatory bowel disease (IBD) patients. Gastroenterology 2013;144:S633–4.

Publication IV

sciendo

PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B, Vol. 76 (2022), No. 1 (736), pp. 20–30.

DOI: 10.2478/prolas-2021-00XX

BODY MUSCLE MASS METABOLIC DATA ANALYSIS IN ASSOCIATION WITH CROHN'S DISEASE ACTIVITY

Polina Zaļizko[1,2,3,#], Monta Urbāne[1,3], Terēze Hermīne Roshofa[3], Viktorija Mokricka[1,2], Laila Meija[1,3], Edgars Bodnieks[1,3], and Aldis Puķītis[1,2]

[1] Pauls Stradiņš Clinical University Hospital, 13 Pilsoņu Str., Riga, LV-1002, LATVIA

[2] Faculty of Medicine, University of Latvia, 19 Raina Blvd., Riga, LV-1586, LATVIA

[3] Faculty of Medicine, Rīga Stradiņš University, 16 Dzirciema Str., Riga, LV-1007, LATVIA

[#] Corresponding author, polina.zalizko@stradini.lv

Communicated by Dainis Krieviņš

Malnutrition is a common complication of Crohn's disease (CD) patients and it is correlated with alterations of the body composition and disease activity. Our prospective pilot study included hospitalised CD patients, age ≥18 years. Patients were assessed using the Nutritional Risk Score (NRS2002), the Malnutrition Universal Screening Tool (MUST), and body bioelectrical impedance analysis. Twenty-three hospitalised patients (median age 36.5, interquartile range (IQR): 28.5–51.5 years) were enrolled; the median CD activity index was 128 (IQR = 6.0–207.0). The study group comprised 48% (n = 11) patients with low CD activity and 52% (n = 12) with high disease activity. According to NRS2002 and MUST, 70% (n = 16) CD patients had malnutrition risk and were in need of nutritional support. The median BMI was lower for the CD group (21.10 [IQR = 19.2–23.3]) than for the control group (23.4 [IQR = 21.5–25.8]) (p = 0.014). In terms of deviation from standard weight, 39% (n = 9) of CD patients showed reduced % body fat. Reduced muscle mass was observed in 48% (n = 11) of CD patients. CD patients with high disease activity had a noticeably increased risk of malnutrition. Identification of the reduction in soft lean muscle mass in CD patients can be used as an anticipatory indicator of disease activity.

Key words: *inflammatory bowel disease, Crohn's disease activity index, malnutrition, screening tools.*

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) with periods of activity and remission (Ferre *et al.*, 2018). In industrialised countries, IBD has become a global disease and the incidence of CD has risen significantly over the past half century (Levine *et al.*, 2020). The highest reported prevalence values were in Europe — 322 cases per 100 000 (Ng, 2017). Smoking, use of antibiotics, and diet are potentially preventable risk factors for IBD (Forbes *et al.*, 2017). CD commonly can lead to malnutrition, estimated in 65–75% of patients with CD (Scaldaferri *et al.*, 2017), and specific nutritional deficiencies, which may be caused by low dietary intake, changes in metabolism, increased intestinal protein loss, and nutrient malabsorption (Jahnsen *et al.*, 2003; Wędrychowicz *et al.*, 2016). The nutritional status of IBD patients is frequently altered, even when the disease is in remission, although it is directly related to the severity of the disease (Back *et al.*, 2017; Casanova *et al.*, 2017).

Malnutrition is an objective disease activity parameter for patients with IBD, particularly CD, and is an indicator of systemic damage or inflammatory activity. Active disease is correlated with the systemic response of the body's immune system, activating a hypermetabolic state and protein degradation, decreasing protein synthesis (Argiles *et al.*, 2015). These conditions lead to malnutrition, which significantly increases the risk of impaired clinical outcomes, such as delayed recovery or increased mortality (Landi *et al.*, 2019). Inadequate body composition and malnutrition have been associated with poor outcomes, such as a higher frequency of postoperative complications, longer hospital stays, decreased quality of life and higher health costs (Casanova *et*

al., 2017). The severity of malnutrition depends on the activity, duration and extent of the disease, and inflammatory response, which drives catabolism (Forbes *et al*., 2017).

To evaluate malnutrition, basic anthropometry techniques were used, such as body mass index (BMI) and biochemical parameters, but they are not accurate enough to estimate body composition. In a high proportion of IBD patients, these values may be within normal ranges, while they still have an altered body composition. Therefore, bioelectrical impedance analysis (BIA) is used to calculate total body water (TBW), and to estimate fat-free mass (FFM) [lean mass], muscle and fat mass (Casanova *et al*., 2017). BIA is easy, non-invasive, relatively inexpensive and can be performed in almost any subject because it is portable (Kyle, *et al*., 2004). Screening for malnutrition, using screening tools like the Nutritional Risk Screening Score 2002 (NRS2002) and Malnutrition Universal Screening Tool (MUST), which are recommended by the European Society of Clinical Nutrition and Metabolism, is important to identify subjects at nutritional risk (Kondrup *et al*., 2003). Referring to recommendations, screening should be performed within the first 24–48 h after first contact and thereafter at regular intervals. For those identified as being at risk by nutritional screening, nutritional assessment should be provided (Caderholm *et al*., 2017).

Recent studies have shown that between 22% and 60% of patients with IBD have FFM depletion. This is significant because FFM depletion has been associated with negative outcomes, including major postoperative complications predicting a small bowel resection, primary non-response to antitumor necrosis factor (TNF) agents, and osteopenia. Traditional nutritional measurements, like BMI, correlate poorly with indices of FFM in patients with CD, resulting in a risk for under recognition and underestimation of the extent of nutrition depletion when relying only on weight-based indicators of nutritional status (Wood, 2020). Reduced muscle mass has been included in the Global Consensus for Diagnosing Malnutrition in Adult Patients (Cedeholm *et al*., 2019).

The aim of this study was to analyse body muscle mass metabolic data in association with Crohn's disease, identifying patients with low and high disease clinical activity.

MATERIALS AND METHODS

This prospective pilot study included twenty-three hospitalised patients aged ?18 years, with an established diagnosis of CD and no medical history of surgical interventions. CD patients were divided into those with low and high clinical activity indexes, according to the CD activity index (CDAI): low clinical activity index (CDAI < 150) and high clinical activity index (CDAI > 150).

Patients were assessed twice using the Nutritional Risk Score (NRS2002) and Malnutrition Universal Screening Tool (MUST), recommended by the European Society of Clinical Nutrition and Metabolism. The MUST has been found to have excellent inter-rater reliability, concurrent validity with other tools, and predictive validity (length of hospital stay and mortality). Its purpose is to identify individuals at risk of developing malnutrition based on nutrition status (BMI and weight loss) and disease-related dysfunction. NRS2002 contains the nutritional components of MUST, and in addition, a grading of severity of disease as a reflection of increased nutritional requirements; its purpose is to identify patients at risk of malnutrition within 48 hours after hospital admission and to determine those who would benefit from early nutrition therapy (Kondrup *et al*., 2003; Rabito *et al*., 2017).

Body bioelectrical impedance analysis (BIA) measurements were performed using impedance equipment at least three hours after eating, as this may reduce small errors in impedance determination (Kyle *et al*., 2004). The whole body was measured, by subdividing it into various segments. Furthermore, weight (kg), BMI, fat-free mass (FFM) (kg), soft lean mass (SLM) (kg), metabolic body fat (MBF) (kg), total body water (TBW) (kg), basal metabolic rate (kcal), total energy expenditure (kcal), visceral fat (%), and per cent body fat (PBF) were assessed.

A control group consisting of twenty-three healthy age- and sex-matched individuals was used for comparison. BIA was performed if the person met the inclusion criteria: age ?18 years old and age-matched to the patient group, with no food intake ?3 hours prior to testing, and with no nutritional risks identified using both screening tools (NRS2002 and MUST), which assured that the control group did not harbour any active disease that would interfere with the study results.

Statistical analysis was performed using SPSS version 22 (IMDB Statistical Package for the Social Sciences 22). Statistically significance was set at *p*-values ≤ 0.05. The study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee.

RESULTS

Twenty-three hospitalised patients (median age 36.5, interquartile range (IQR): 28.5–51.5 years) were enrolled, 39% (n = 9) were female and 61% (n = 14) male. The median CDAI was 128 (IQR = 6.0–207.0). The study group comprised 48% (n = 11) patients with low CD activity and 52% (n = 12) of patients with high disease activity (Fig. 1).

According to NRS2002 and MUST, 70% (n = 16) CD patients were nutritionally at risk and in need of nutritional support. From twelve patients with high disease activity, 92% (n = 11) had a risk of malnutrition, and from eleven patients with low disease activity, only 45% (n = 5) has a risk of malnutrition ($p = 0.027$). Clinical nutrition was administered to 11 patients; enteral oral feeding was prescribed to nine of these patients. Additionally, two patients received parenteral peripheral feeding.

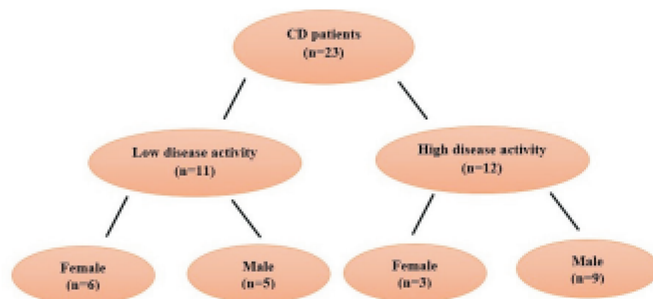


Fig. 1. Diagram of study flow.

Table 1. Comparison of parameters between Crohn's disease patients and the control group

Parameters	CD patients	Control group	*p*-value
Weight (kg)	68.7 _14.8	73.5 _13.9	0.232
PBF (%)	21.3 _7.5	21.8 _9.9	0.355
Muscle mass deviation from normal	0.6 _3.1	2.9 _4.6	0.143
Fat deviation from normal	1.3 _6.8	1.8 _5.0	0.122
Muscle mass	49.6 [41.1–57.3]	55.8 [42.9–61.4]	0.425
Proteins	38.7 [32.2–44.4]	12.5 [9.6–13.8]	< 0.001
Minerals	3.8 [3.4–4.7]	4.3 [3.6–4.7]	0.212
TBW	38.7 [32.20–44.35]	43.1 [33.4–47.4]	0.454
Basal metabolism	1503.0 [1196.00–1607.0]	1518.5 [1266.5–1711.8]	0.407
Weight deviation from normal	4.1 [–8.4–6.3]	4.2 [–1.2–12.2]	0.102
BMI	23.4 [19.2–23.3]	21.1 [21.5–25.8]	0.014
Visceral fat %	–4.0 [–12.1–5.6]	7.9 [–0.9–18.2]	0.003

BMI, body mass index; CD, Crohn's Disease; PBF, percent body fat; TBW, total body water

Data obtained by BIA was compared between the study group to those of the population control group (Table 1). Statistically important differences were observed in BMI, % visceral fat and proteins. Other values also seemed to vary, but analysis did not show statistically significant differences. The albumin level was determined for17 patients, of whom 24% (n = 4) had a reduced albumin level (.5 g/dl) at the moment of admission.

Comparing BIA results between CD patients and the control group, the median BMI was significantly lower for the CD (21.10 [IQR = 19.2–23.3]) than for the control group (23.4 [IQR = 21.5–25.8]) (p = 0.014). In addition, visceral fat mass was significantly lower in the CD (–4.00 [IQR = –12.1 to 5.6]) than in the control group (7.85 [IQR = –0.9–18.2]) (p = 0.003). An imbalanced body composition with changes in PBF and SLM was observed in a large proportion of patients. In terms of deviation from standard weight, 39% (n = 9) of CD patients showed reduced PBF. This indicated that even though patients had BMI within normal values, there was a high chance of having imbalance in body composition. Reduced muscle mass was observed in 48% (n = 11) of CD patients. CD patients were more prone to reduction in muscle mass when disease activity is high. Only a few individuals of the control group had muscle mass under the normal values.

DISCUSSION

In our study, according to NRS2002 and MUST, 70% CD patients were nutritionally at risk and in need of nutritional support. In a previous study (Stratton *et al.*, 2004) using MUST, the prevalence of malnutrition risk ranged from 19–60% in inpatients and 30% in outpatients. Our results were higher, which can be explained by higher disease activity and probably with various nutritional and metabolic disturbances. Our data indicated strong agreement between both scales on evaluation of the nutritional status of the patient. Similar agreement has also been reported (Stratton *et al*. 2004), where MUST had excellent agreement (k 0·775–0·893) with MEREC, NRS and SGA tools).

Those identified to be at risk of malnutrition by using malnutrition screening tools, should be followed by nutritional assessment, which will give the basis for the diagnosis, as well as for further action including nutritional treatment. Assessment of the nutritional status integrates information on body weight, body height, body mass index (kg/m^2), body composition and biochemical indices (Cederholm *et al.*, 2017).

Malnutrition is defined as a subacute or chronic state in which a combination of negative energy balance and varying degrees of inflammatory activity has led to changed

body composition, diminished function, and adverse outcomes (Cederholm *et al*., 2017). Malnutrition adversely affects physical and psychological function and impairs a patients recovery from disease and injury, thereby increasing morbidity and mortality. Despite being a common problem, malnutrition is frequently unrecognised and untreated in many health care settings, including nursing and other care homes, general practice, and hospital outpatients and inpatients (Stratton *et al*., 2004).

It has been reported that ileal involvement in CD patients plays a relevant role in reducing nutrient absorption (Balestrieri *et al*., 2020). Nutritional deficiencies are self-evidently more likely in patients with CD affecting the small bowel than in those with isolated colonic disease (Forbes *et al*., 2017).

The severity of malnutrition is dependent on the activity, duration, and extent of the disease and, in particular, on the magnitude of the inflammatory systemic response mediated by pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukins-1 and -6, which can increase catabolism and lead to anorexia (Balestrieri *et al*., 2020). In our study it was confirmed that the patients with higher disease activity has a higher risk of malnutrition. From twelve patients with high disease activity, 92% (n = 11) had a risk of malnutrition, and from eleven patients with low disease activity, only 45% (n = 5) had a risk of malnutrition. This difference was statistically significant (p = 0.027).

Detection of reduced body muscle mass has emerged as a crucial variable in nutritional assessment. A progressive and general loss of lean muscle mass associated with decreased muscle strength or physical performance has significant impact on quality of life and causes physical disability (Balestrieri *et al*., 2020). A systematic review (Ryan *et al*., 2019) reported that up to 60% IBD patients have decreased muscle mass when compared with healthy subjects and a higher percentage were patients with CD and these patients were more likely to be male (Ryan *et al*., 2019). In our study, reduced muscle mass was observed in 48% of CD patients and 39% were men. Low lean mass is likely a surrogate marker of ill-health and inadequately controlled disease activity, and may be associated with fatigue and reduced quality of life in patients with IBD. Some studies have shown that BMI does not correlate well with lean mass in patients with IBD, even amongst those in clinical remission. Increase of BMI may be related to gain in fat mass, further masking the underlying lean mass deficit. BMI was shown to better correlate with fat mass than with lean mass. In fact, a normal BMI was falsely reassuring in 72% of patients who were demonstrated to have low lean mass (Bryant *et al*., 2015).

According to the World Health Organisation standardised criteria, patients were considered to be well-nourished when their BMI was between 18.5 and 24.5 kg/m^2, underweight or malnourished when their BMI was ≤ 18.5 kg/m^2, and overweight when their BMI was ≥ 25 kg/m^2. According our results, BMI was decreased only in three patients. Therefore, the evaluation of BIA parameters is important. Evidence shows that reduced muscle mass can also be related to an unchanged or even elevated BMI (Balestrieri *et al*., 2020). Therefore, it is important to assess those who are not visibly malnourished.

In systematic review (Ryan *et al*., 2019), it was reported that the serum albumin concentration was significantly lower in patients with CD compared with controls (2.6 g/dl vs 3.0 g/dl; p = 0,002). In another review (Zhang *et al*., 2017) the estimated mean albumin concentration for patients at high risk of malnutrition (detected by NRS-2002) was 3.42 (95% CI: 3.19, 3.64). This indicates that using albumin with a cut-off of 3.5 g/dl would fail to identify a proportion of the patients diagnosed to be at high risk of malnutrition using NRS-2002, not to mention those at low malnutrition risk. In our study, from seventeen patients for whom the albumin level was determined, only four patients had a reduced albumin level (< 3.5 g/dl). Therefore, the significance of serum albumin as an indicator of nutritional status is controversial, because the serum albumin level is affected by intravenous fluids and dehydration (Takaoka *et al*., 2018).

Monitoring of anthropometry provides insight into which patients develop relative deficits in lean mass and therefore would benefit from nutritional supplementation. The protein requirement is increased in active disease, and intake should be increased, but in remission the protein requirements are generally not elevated and provision should be similar to that recommended in the general population. In general, no specific diet needs to be followed during remission phases (Forbes *et al*., 2017)

Nutrition or nutrients can be provided orally, via enteral tube-feeding or as parenteral nutrition to prevent or treat malnutrition in an individualised way (Cederholm *et al*., 2017). The nutritional care plan is based on the results of the assessment. This plan should be developed by a multi/interdisciplinary team together with the patient. In our study, nine patients received enteral and two patients parenteral feeding, that was individualised for each patient based on nutritional assessment results.

CONCLUSION

CD patients with high disease activity had a noticeably increased risk of malnutrition. 48% of CD patients in both the low and high disease activity groups had a reduction in muscle mass. Identification of the reduction in soft lean muscle mass in CD patients can be used as an indicator of disease activity.

REFERENCES

Argilés, J. M., Busquets, S., Stemmler, B., López-Soriano, F. J. (2015). Cachexia and sarcopenia: Mechanisms and potential targets for intervention. *Curr. Opin. Pharmacol*., **22**, 100–106.

Back, I. R., Marcon, S. S., Gaino, N. M., Vulcano, D. S. B., Dorna, M. S., Sassaki, L. Y. (2017). Body composition in patients with Crohn's disease and ulcerative colitis. *Arq. Gastroenterol.*, **54** (2), 109–114.

Balestrieri, P., Ribolsi, M., Guarino, M. P. L., Emerenziani, S., Altomare, A., Cicala, M. (2020). Nutritional aspects in inflammatory bowel diseases. *Nutrients*, **12** (2), 372.

Bryant, R. V. https://pubmed.ncbi.nlm.nih.gov/25753216/ - affiliation-1, Ooi, S., Schultz, C. G., Goess, C., Grafton, R., Hughes, J., Lim, A., Bartholomeusz, F. D., Andrews, J. M. (2015). Low muscle mass and sarcopenia: Common and predictive of osteopenia in inflammatory bowel disease. *Aliment Pharmacol. Ther. Actions*, **41** (9), 895–906.

Casanova, M. J., Chaparro, M., Molina, B., Merino, O, Batanero, R., Duenas-Sadornil, C., Robledo, P., Garcia-Albert, A. M., Gomez-Sanchez, M. B., Calvet, X., *et al.* (2017). Prevalence of malnutrition and nutritional characteristics of patients with inflammatory bowel disease. *J. Crohns. Colitis*, **11** (12), 1430–1439.

Cederholm, T., Barazzoni, R., Austin, P., Ballmer, P., Biolo, G., Bischoff, S. C., Compher, C., Correia, I., Higashiguchi, T., Holst, M., *et al.* (2017). ESPEN guidelines on definitions and terminology of clinical nutrition. *Clin. Nutr.*, **36** (1), 49–64.

Ferré, M. P. B., Boscj-Watts, M. M., Pérez, M. M. (2018). Crohn's disease. *Med. Clin. (Barc).*, **151** (1), 26–33.

Forbes, A., Escher, J., Hébuterne, X., Kłęk, S., Krznaric, Z., Schneider, S., Shamir, R., Stardelova, K., Wierdsma, N., Wiskin, A. E., Bischoff, S. C. (2017). ESPEN guideline: Clinical nutrition in inflammatory bowel disease. *Clin. Nutr.*, **36** (2), 321–347.

Jahnsen, J. Falch, J. A., Mowinckel, P., Aadland, E. (2003). Body composition in patients with inflammatory bowel disease: A population-based study. *Amer. J. Gastroenterol.*, **98** (7), 1556–1562.

Kondrup J., Allison, M., Elia, M., Vellas, B., Plauth, M. (2003). ESPEN Guidelines for Nutrition Screening. *Clin. Nutr.*, **22** (4), 415–421.

Kyle, U. G., Bosaeus, I., De Lorenzo, A. D., Deurenberg, P., Elia, M., Gómez, J. M., Heitmann, B. L., Kent-Smith, L., Melchior, J. C., Pirlich, M., Scharfetter H. (2004). Bioelectrical impedance analysis. Part II: Utilization in clinical practice. *Clin. Nutr.*, **23** (6), 1430–1453.

Landi, F., Camprubi-Robles, M., Bear, D. E., Cederholm, T., Malafarina, V., Welch, A. A., Cruz-Jentoft, A. J. (2019). Muscle loss: The new malnutrition challenge in clinical practice. *Clin. Nutr.*, **38** (5), 2113–2120.

Levine, J. S., Burakoff, R. (2016). Inflammatory bowel disease: Medical considerations. In: Greenberger, N. J., Blumberg, R. S., Burakoff, R. (eds.). *CURRENT Diagnosis & Treatment: Gastroenterology, Hepatology & Endoscopy*. 3rd edition. McGraw-Hill. 656 pp.

Ng, S. C., Shi, H. Y., Hamidi, N., Underwood, F. E., Tang, W., Benchimol, E. I., Panaccione, R., Ghosh, S., Wu, J. C., Chan, F. K. L., Sung, J. J. Y., Kaplan, G. G. (2018). Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: A systematic review of population-based studies. Lancet, **390** (10114), 2769–2778.

Rabito, E. I., Marcadenti, A., Fink, J. S., Figueira, L., Silva, F. M. (2017). Nutritional risk screening 2002, Short Nutritional Assessment Questionnaire, Malnutrition Screening Tool, and Malnutrition Universal Screening Tool are good predictors of nutrition risk in an emergency service. *Nutr. Clin. Pract.*, **32** (4), 526–532.

Ryan, E., McNicholas, D., Creavin, B., Kelly, M. E., Walsh, T., Beddy D. (2019). Sarcopenia and inflammatory bowel disease: A systematic review. *Inflamm. Bowel Dis.*, **25** (1), 67–73.

Scaldaferri F., Pizzoferrato M., Lopetuso L.R., Musca T., Ingravalle F., Sicignano L.L., Mentella M., Miggiano G., Mele M.C., Gaetani E., *et al.* (2017). Nutrition and IBD: Malnutrition and/or sarcopenia? A practical guide. *Gastroenterol. Res. Pract.*, **2017**, 8646495.

Stratton, R. J., Hackston, A., Longmore, D., Dixon, R., Price, S., Stroud, M., King, C., Elia, M. (2004). Malnutrition in hospital outpatients and inpatients: Prevalence, concurrent validity and ease of use of the 'malnutrition universal screening tool' ('MUST') for adults. *Brit. J. Nutr.*, **92** (5), 799–808.

Takaoka, A., Sasaki, M., Nakanishi, N., Kurihara, M., Ohi, A., Bamba, S., Andoh, A. (2018). Nutritional screening and clinical outcome in hospitalized patients with Crohn's disease. *Ann. Nutr. Metab.*, **71**, 266–272.

Wędrychowicz, A., Zając, A., Tomasik P. (2016). Advances in nutritional therapy in inflammatory bowel diseases: Review. *World J. Gastroenterol.*, **22** (3), 1045–1066.

Wood, J., Ward, L., Sparrow, M., King, S. (2020). Utility of bioimpedance methods for the assessment of fat-free mass in adult outpatients with inflammatory bowel disease. *Nutrition*, **77**, 110833.

Zhang, Z., Pereira, S. L., Luo, M., Matheson, E. M. (2017). Evaluation of blood biomarkers associated with risk of malnutrition in older adults: A systematic review and meta-analysis. *Nutrients*, **9** (8), 829.

Received 22 March 2021
Accepted in the final form 4 January 2022

ĶERMEŅA MUSKUĻU MASAS METABOLO RĀDĪTĀJU ANALĪZE SAISTĪBĀ AR KRONA SLIMĪBAS AKTIVITĀTI

Krona slimība ir hroniska iekaisīga zarnu slimība ar slimības aktivitātes un remisijas periodiem. Iekaisīgas zarnu slimības ir kļuvušas par globālu problēmu. Krona slimības izplatība ievērojami pieaugusi pēdējo gadu laikā. Malnutrīcija ir bieži sastopama komplikācija Krona slimības pacientiem. Tā korelē ar slimības aktivitāti un izmaiņām ķermeņa kompozīcijā. Pārmaiņas ķermeņa kompozīcijā ir saistītas ar biežākām pēcoperācijas komplikācijām, ilgāku hospitalizāciju, samazinātu dzīves kvalitāti un augstākām veselības aprūpes izmaksām. Šī pētījuma mērķis bija analizēt ķermeņa muskuļu masas vielmaiņas radītāju saistību ar Krona slimību un zemu vai augstu slimības aktivitāti. Pētījumā iekļauti 23 hospitalizēti Krona slimības pacienti vecumā ?18 gadi. Pacienti tika novērtēti divas reizes, izmantojot malnutrīcijas skrīninga skalas *Nutritional Risk Score* (*NRS* 2002) un *Malnutrition Universal Screening Tool* (*MUST*), kā arī tika veikta ķermeņa bioelektriskās impedances analīze. Pētījumā novērots, ka pacientiem ar augstu slimības aktivitāti bija ievērojami augstāks malnutrīcijas risks. Lielai daļai (48%) Krona slimības pacientu gan ar zemu, gan augstu slimības aktivitāti bija samazināta muskuļu masa. Muskuļu masas samazinājuma noteikšanu Krona slimības pacientiem var pielietot kā slimības aktivitātes rādītāju. Pētījumā konstatēts, ka ne tikai pacientiem ar samazinātu ķermeņa masas indeksu (ĶMI), bet arī pacientiem ar normālu vai paaugstinātu ĶMI ir augsts ķermeņa kompozīcijas izmaiņu risks.

Latvian patent Nr. LV 15508 B1

LV 15508

(19)

(11) **LV 15508 B1**

(51) Starpt.pat.kl. **C12Q1/48**
C12Q1/6827
C12Q1/686

Latvijas patents izgudrojumam
2007g. 15.februāra Latvijas Republikas likums

(12) **Īsziņas**

(21) Pieteikuma numurs: P-18-96	**(71) Īpašnieks(i):** LATVIJAS UNIVERSITĀTE,Raiņa bulvāris 19,Rīga,LV
(22) Pieteikuma datums: 05.12.2018	**(72) Izgudrotājs(i):** Aldis PUĶĪTIS (LV), Jeļizaveta SOKOLOVSKA (LV), Juris STEFANOVIČS (LV), Poļina ZAĻIZKO (LV)
(43) Pieteikuma publikācijas datums: 20.06.2020	**(74) Pilnvarnieks vai pārstāvis:** Aleksandra FORTŪNA, FORAL Intelektuālā īpašuma aģentūra, SIA,Kalēju iela 14 - 7,Rīga,LV
(45) Patenta publikācijas datums: 20.02.2021	

(54) Izgudrojuma nosaukums: PAŅĒMIENS TIOPURĪNA METILTRANSFERĀZES POLIMORFISMU NOTEIKŠANAI
METHOD OF DETECTION OF THIOPURINE METHYLTRANSFERASE POLYMORPHISMS

(57) Kopsavilkums:

Izgudrojums attiecas uz paņēmieniem un vielām tiopurīna metiltransferāzes polimorfismu (TPMT) noteikšanai. Paņēmiens TPMT polimorfismu noteikšanai ietver šādus secīgus soļus: (i) bioloģiska parauga, kas satur pacienta DNS, nodrošināšana; (ii) pirmā maisījuma, kas satur DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1800462 (TPMT*2) noteikšanai pielāgotus praimerus un divas hidrolīzes zondes, nodrošināšana; (iii) otrā maisījuma, kas satur DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1800460 (TPMT*3B) noteikšanai pielāgotus praimerus un divas hidrolīzes zondes, nodrošināšana; (iv) trešā maisījuma, kas satur DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1142345 (TPMT*3C) noteikšanai pielāgotus praimerus un divas hidrolīzes zondes, nodrošināšana; (v) polimerāzes ķēdes reakcijas (PĶR) premiksa nodrošināšana; (vi) no bioloģiskā parauga izdalītās DNS un premiksa pievienošana pirmajam, otrajam un trešajam maisījumam kvantitatīvās PĶR veikšanai un mērķa nukleīnskābes amplifikācijai; (vii) fluorescences mērīšana un TPMT gēna polimorfismu noteikšana, salīdzinot amplifikācijas rezultātus ar iepriekš zināmajiem parauglielumiem.

IZGUDROJUMA APRAKSTS

Tehnikas joma

[001] Izgudrojums attiecas uz paņēmieniem un vielām tiopurīna metiltransferāzes polimorfismu noteikšanai.

Iepriekšējais tehnikas līmenis

[002] Tiopurīna metiltransferāze (TPMT) ir enzīms, kas organismā katalizē S-metilācijas procesu un nodrošina citostatisku medikamentu metabolizēšanos neaktīvā metilētā formā pacientiem, kas lieto tiopurīnu grupas ārstnieciskos līdzekļus. Neskatoties uz farmakoloģisko TPMT nozīmi metabolismā, tās funkcija organismā līdz galam nav izpētīta.
Pastāv vairāki TPMT ģenētiski polimorfismi, kas ietekmē šī enzīma aktivitāti un var būt par iemeslu tiopurīnu grupas medikamentu toksicitātei, kura var izraisīt cilvēka dzīvībai bīstamas blakus reakcijas (piemēram, mielosupresiju).

[003] Lai izvērtētu tiopurīnu grupas medikamentu toksicitāti un izvēlētos optimālu medikamentu devu, ir jānovērtē TPMT enzīma aktivitāte, nosakot izplatītākos TPMT aktivitāti ietekmējošos polimorfismus visiem pacientiem, kuriem paredzēta terapija ar tiopurīnu grupas medikamentiem (piemēram, azatioprīnu un merkaptopurīnu).

[004] Ir zināms paņēmiens TPMT aktivitātes analīzei *in vitro*, pielietojot *urothione* un/vai *jukathione* koncentrācijas noteikšanu cilvēka organisma bioloģiskos šķidrumos (WO2015007666).

[005] Ir zināms paņēmiens TPMT aktivitātes noteikšanai eritrocītos (WO03020961) pacientiem pirms azatiopurīna terapijas uzsākšanas. Paņēmiens ietver pacienta TPMT saturoša eritrocītu parauga inkubāciju, pievienojot pētāmam substrātam metilgrupas donoru. Inkubācija notiek apstākļos, kuros TPMT enzīms katalizē reakcijas produktu. Metabolītus ekstraģē ar etilacetātu, izmantojot šķidrumu hromatogrāfijas metodi, nosaka to koncentrāciju.

[006] Ir zināms paņēmiens TPMT aktivitātes noteikšanai, kas ietver sekojošus soļus: (i) asins parauga pievienošana TPMT metildonoram (S-adenozilmetionīnam) un references vielai

(izotopiski marķētam TPMT – metilētam TPMT) substrātam reakcijas maisījuma veidošanai; (ii) iegūtā maisījumu izdalīšana ar organisku šķīdinātāju ekstrahētas frakcijas veidošanai, kas satur references vielu un iegūto metilēto TPMT metabolītu; (iii) references vielas un metilēta TPMT metabolīta koncentrāciju ekstrahētajā frakcijā nosaka, izmantojot šķidrumu hromatogrāfiju tandēmā ar masspektrometriju (US7452689).

[007] Jau zināmajiem paņēmieniem ir virkne trūkumu - rezultātus var ietekmēt daudzi ārēji faktori. Ja pacients saņem medikamentus, kas inhibē TPMT enzīma aktivitāti, vai pacientam ir iepriekš veiktas hemotransfūzijas, vai pacients jau lieto kādu no tiopurīnu grupas medikamentiem, tad ar šo zināmo paņēmienu nevar iegūt ticamu rezultātu.

[008] Ir zināms paņēmiens TPMT genotipa noteikšanai ar kvantitatīvās polimerāzes ķēdes reakcijas (PĶR) metodi, izmantojot fluorescentās hidrolīzes zondes (Marzena Skrzypczak-Zielinska et.al. *A Simple Method for TPMT and ITPA Genotyping Using Multiplex HRMA for Patients Treated with Thiopurine Drugs*. – Molecular Diagnosis & Therapy 2016; 20(5): 493-499). Zondes saistās ar kvantitatīvās PĶR pavairotajām DNS kopijām TPMT gēna vietās, kas var saturēt polimorfismus. Polimerāzei sašķeļot zondes, tiek emitēts fluorescents signāls, kas tālāk tiek kvantitatīvi izmērīts un, attiecīgi, salīdzinot ar references datiem, tiek izdarīti secinājumi par tiopurīna metiltransferāzes polimorfismu. Aprakstītā paņēmiena galvenais trūkums ir tā salīdzinoši lielais reaktīvu patēriņš (pašizmaksa).

Izgudrojuma izklāsts

[009] Izgudrojuma mērķis ir piedāvāt alternatīvu, izmaksu un patērētā laika ziņā efektīvu tiopurīna metiltransferāzes polimorfismu noteikšanas paņēmienu. Izvirzītais mērķis tiek sasniegts ar piedāvāto paņēmienu, kas ietver šādus soļus: (i) bioloģisku paraugu, kas satur pacienta DNS, nodrošināšana; (ii) pirmā maisījuma nodrošināšana, kas satur praimerus un divas hidrolīzes zondes, kuras attiecīgi ir pielāgotas DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1800462 (TPMT*2) noteikšanai; (iii) otrā maisījuma nodrošināšana, kas satur praimerus un divas hidrolīzes zondes, kuras attiecīgi ir pielāgotas DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1800460 (TPMT*3B) noteikšanai; (iv) trešā maisījuma nodrošināšana, kas satur praimerus un divas hidrolīzes zondes, kuras attiecīgi ir pielāgotas DNS mērķa sekvences

pavairošanai un TPMT gēna polimorfisma rs1142345 (TPMT*3C) noteikšanai; (v) PĶR premiksa nodrošināšana; (vi) no bioloģiskā parauga izdalītās DNS un premiksa pievienošana pirmajam, otrajam un trešajam maisījumam kvantitatīvai polimerāzes ķēdes reakciju (PĶR) veikšanai un mērķa nukleīnskābes PĶR amplifikācijai; (vii) fluorescences mērīšana un TPMT gēna polimorfismu noteikšana, salīdzinot amplifikācijas rezultātus ar iepriekš zināmajiem parauglielumiem.

[010] References laboratorijā veiktās DNS un kvantitatīvās PĶR pozitīvo paraugu pārbaude ar alternatīvām molekulārās bioloģijas metodēm (restrikcijas fragmentu garuma polimorfisma analīzi un alēlspecifisko PĶR) pierāda augstu izgudrotāju aprakstītās kvantitatīvās PĶR metodes precizitāti. Salīdzinot ar tuvākajiem analogiem, piedāvātais izgudrojums nodrošina ātrāku un izmaksu ziņā efektīvu tiopurīna metiltransferāzes polimorfismu noteikšanu.

[011] Piedāvātais paņēmiens ir izmantots vairāk nekā 250 pacientu DNS paraugu *in vitro* testēšanā ar *Applied Biosystems StepOnePlus* analizatoru LU Medicīnas fakultātes Personalizētās medicīnas laboratorijā. Salīdzinot ar tuvāko analogu, izgudrojums paredz zemāku hidrolīzes zondes koncentrāciju reakcijas maisījumā, mazāku reakcijas tilpumu un citu reaģentu daudzumu. Piedāvātajā paņēmienā ir iespējams izmantot dažādu ražotāju *Master Mix*, kas neietekmē galarezultātu. Kombinējot dažādu ražotāju standarta reaģentus, var iegūt ekonomiski izdevīgāku rezultātu. Ar piedāvāto paņēmienu tiek panākts ātrāks rezultāts īsākā PĶR laikā.

[012] Saskaņā ar izgudrojumu TPMT gēna polimorfismu noteikšanai tiek piedāvāts izmantot divus PĶR reakcijas maisījumu sastāvus. Pirmais maisījuma sastāvs satur PĶR premiksu (2x), savukārt otra maisījuma sastāvā ir salīdzinoši lētāks PĶR premikss (5x); abu maisījumu sastāvi ir optimizēti trīs TPMT gēna polimorfismu noteikšanai: rs1800462 (TPMT*2), rs1800460 (TPMT*3B), rs1142345 (TPMT*3C). Pētījumos ir pierādīts, ka minētās gēna polimorfisma variācijas būtiski samazina TPMT aktivitāti, dažos gadījumos pilnībā inaktivē enzīmu un korelē ar tiopurīna grupas medikamentu toksicitāti, kas var izraisīt cilvēka dzīvībai bīstamas blakus reakcijas.

[013] TPMT genotipa noteikšanu veic ar kvantitatīvo polimerāzes ķēdes reakcijas (PĶR) metodi, izmantojot fluorescentās hidrolīzes zondes. Zondes saistās ar PĶR reakcijā pavairotajām DNS

kopijām TPMT gēna vietās, kas var saturēt polimorfismus. Polimerāzei sašķeļot zondes, tiek emitēts fluorescents signāls. Stobriņus ar PĶR reakcijas maisījumiem ievieto reālā laika PĶR iekārtā un veic kvantitatīvo PĶR analīzi. Kvantitatīvās polimerāzes ķēdes reakcijas metodes pamatā ir DNS amplifikācija, ko veic termostabila polimerāze.

[014] Saskaņā ar piedāvāto izgudrojumu DNS pavairošanu veic 40 DNS sintēzes ciklos. Reakcijas eksponenciālajā fāzē ar katru nākamo ciklu DNS daudzums pieaug divas reizes. Katru sintēzes ciklu veido trīs reakcijas posmi: DNS denaturācija, praimeru hibridizācija (piesaistīšanās) un DNS sintēze.

[015] Pirmajā posmā notiek sākotnējā denaturācija jeb DNS komplementāro ķēžu atdalīšana un polimerāzes aktivēšana. 95 °C temperatūrā noārdās ūdeņraža saites starp slāpekļa bāzēm un izveidojas divas vienpavediena DNS ķēdes.

[016] Nākamajā posmā notiek praimeru piesaistīšanās pie vienpavediena DNS. Praimeri satur aptuveni 20 – 30 bāzu pārus (vienpavediena DNS fragmentus), turklāt katrs no praimeriem piesaistās savai DNS vienpavediena molekulai tā, lai katrs no savas puses ietvertu pavairojamo DNS fragmentu. Praimeru sekvence ir specifiski piemērota tam, lai piesaistītos noteiktā DNS rajonā. Piesaistīšanās temperatūra parasti ir 50–65 °C. Kvantitatīvā PĶR arī zondes piesaistās pie amplificējamām DNS sekvencēm.

[017] Trešajā posmā polimerāze sāk vienpavediena DNS ķēdēm komplementāri pievienot nukleotīdus, pagarinot praimerus atbilstošā fragmenta virzienā. Sintezētais DNS fragments ir matrice nākamajiem sintēzes cikliem.

[018] Saskaņā ar izgudrojumu, divos reakcijas maisījumos tiek izmantotas hidrolīzes zondes – oligonukleotīdi, kuru molekulas 5' galā ir kovalenti piesaistīta fluoriscējoša viela, bet molekulas otrajā – 3' galā, - fluorescences dzēsējs (*quencher)* – molekula-akceptors, uz kuru tiek pārnesta rezonanses enerģijas no tuvumā esošā fluorofora. Kvantitatīvajā PĶR reakcijā zonde piesaistās pie amplificējamā DNS fragmenta un tiek sašķelta (hidrolizēta), polimerāzei virzoties pa matrices DNS un sastopot ceļā zondi.

19] Zondei hidrolizējoties, fluorofors atšķeļas no enerģijas akceptora, tādēļ fluorescence strauji pieaug. PĶR produkta sintēzes gaitā proporcionāli pieaug nesaistītu fluorescējošu molekulu skaits un ir vērojams kopējais FI pieaugums, ko fiksē PĶR iekārtas sensors.

Izgudrojuma īstenošanas piemēri

[020] Saskaņā ar vēlamo izgudrojuma izpausmi, maisījumu komponenti pirmajam PĶR reakcijas maisījumam tiek izvēlētas šādos daudzumos:

qPĶR premikss (2x)	5 µl
zonde un praimeri	0,125 µl
H_2O	4 µl
DNS	1 µl (3–100 ng)
Kopā	10,125 µl.

[021] Pirmā PĶR maisījuma sastāvā ir komerciāli pieejams PĶR premikss jeb *Master Mix* (5 µl), kas satur visus vajadzīgos komponentus kvantitatīvo PĶR reakciju veikšanai (piemēram, TaqMan ®). Buferšķīduma (2x) sastāvs ar hidrolīzes zondēm ir optimizēts viena nukleotīda polimorfismu noteikšanai. Tā sastāvā ietilpst DNS polimerāze, dNTP, pasīvās references krāsa un citi optimizēti palīgkomponenti ($MgCl_2$, NaN_3, glicerīns). Reakcijas maisījumā ir komerciāli pieejams arī hidrolīzes zonžu un praimeru maisījums (0,125 µl), kurā ietilpst praimeru pāris mērķsekvences pavairošanai (katrs praimeris 18 µM koncentrācijā) un divas hidrolīzes zondes, no kurām viena saistās ar polimorfismu nesaturošu mērķsekvenci, bet otra – ar DNS, kas satur vienu no trim nosakāmajiem polimorfismiem. Maisījumā zondes ir 4 µM koncentrācijā, to 5' galā ir reportieris – fluorofors FAM vai VIC, bet 3'galā fluorescences rezonanses enerģijas akceptors jeb dzēsējs TAMRA. Maisījuma sastāvā ir arī ūdens bez nukleāzēm un DNS matricas.

[022] Maisījumu komponenti otrajam PĶR reakcijas maisījumam tiek izvēlētas šādos daudzumos:

qPĶR premikss (5x)	2 µl
zonde un praimeri	0,125 µl
H_2O	7 µl
DNS	1 µl (3–100 ng)
Kopā	10,125 µl.

[023] Otrā maisījuma sastāvā ir komerciāli pieejams PĶR premikss jeb *Master Mix* (2 µl), kas satur visus vajadzīgos komponentus kvantitatīvo PĶR reakciju veikšanai ar DNS hidrolīzes zondēm (piemēram, SolisByodine ®). Tās ir piemērotas darbam gan ar parastām, gan ar AT vai GC bagātām mērķsekvencēm. Buferšķīdums (5x) satur DNS polimerāzi, dNTP/dUTP (bez UNG), 15 mM $MgCl_2$ un ROX pasīvo references krāsu.

[024] Otrajā PĶR maisījumā tiek izmantots komerciāli pieejams hidrolīzes zonžu un praimeru maisījums (0,125 µl), kurā ietilpst praimeru pāris mērķsekvences pavairošanai (katrs praimeris 18 µM koncentrācijā) un divas hidrolīzes zondes, no kurām viena saistās ar polimorfismu nesaturošu mērķsekvenci, bet otra – pie DNS, kas satur vienu no trim nosakāmajiem polimorfismiem. Maisījumā zondes ir 4 µM koncentrācijā, to 5' galā ir reportieris - fluorofors FAM vai VIC, bet3'galā - fluorescences rezonanses enerģijas akceptors jeb dzēsējs TAMRA. Maisījuma sastāvā ir arī ūdens bez nukleāzēm un DNS matricas.

[025] Saskaņā ar izgudrojumu, polimerāzes ķēdes reakcijas veikšanai tiek izvēlēti šādi režīmi:

<table>
<tr><th>Programmas solis</th><th>Temperatūra</th><th>Laiks</th><th></th></tr>
<tr><td>Sākotnējā denaturācija un polimerāzes aktivēšana</td><td>95 °C</td><td>10 min</td><td></td></tr>
<tr><td>DNS denaturācija</td><td>92 °C</td><td>15 s</td><td rowspan="2">40 cikli</td></tr>
<tr><td>Praimeru hibridizācija un DNS sintēze</td><td>60 °C</td><td>60 s</td></tr>
</table>

PRETENZIJAS

1. Paņēmiens tiopurīna metiltransferāzes (TPMT) polimorfismu noteikšanai, kas ietver šādus secīgus soļus:

(i) bioloģisku paraugu, kas satur pacienta DNS, nodrošināšanu;

(ii) pirmā maisījuma nodrošināšanu, kas satur praimerus un divas hidrolīzes zondes, kurš attiecīgi ir pielāgotas DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1800462 (TPMT*2) noteikšanai;

(iii) otrā maisījuma nodrošināšanu, kas satur praimerus un divas hidrolīzes zondes, kurš attiecīgi ir pielāgotas DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1800460 (TPMT*3B) noteikšanai;

(iv) trešā maisījuma nodrošināšanu, kas satur praimerus un divas hidrolīzes zondes, kurš attiecīgi ir pielāgotas DNS mērķa sekvences pavairošanai un TPMT gēna polimorfisma rs1142345 (TPMT*3C) noteikšanai;

(v) polimerāzes ķēdes reakcijas premiksa nodrošināšanu;

(vi) no bioloģiskā parauga izdalīta DNS un premiksa pievienošanu pirmajam, otrajam un trešajam maisījumam kvantitatīvai polimerāzes ķēdes reakciju (PĶR) veikšanai un mērķa nukleīnskābes PĶR amplifikācijai;

(vii) fluorescences mērīšanu un TPMT gēna polimorfisma noteikšanu, salīdzinot amplifikācijas rezultātus ar iepriekš zināmajiem parauglielumiem.

2. Paņēmiens saskaņā ar 1.pretenziju, turklāt pirmā maisījuma komponenti tiek izvēlēti šādos daudzumos: PĶR premikss (2x) – 5 µl, hidrolīzes zonde un praimeri – 0,125 µl, H_2O – 4µl; turklāt pacienta DNS, kas izdalīta no bioloģiskā parauga, tiek izvēti šādā daudzumā: 1 µl (3–100 ng).

3. Paņēmiens saskaņā ar 1. vai 2. pretenziju, turklāt otrā maisījuma komponenti tiek izvēlēti šādos daudzumos: PĶR premikss (5x) – 2 µl, hidrolīzes zonde un praimeri – 0,125µl, H_2O – 7 µl; turklāt pacienta DNS, kas izdalīts no bioloģiskā parauga, tiek izvēlēta šādā daudzumā: 1 µl (3–100 ng).

4. Paņēmiens saskaņā ar jebkuru no iepriekšminētajām pretenzijām, turklāt kvantitatīvās polimerāzes ķēdes reakcijas veikšanai tiek izvēlēti šādi režīmi:

<table>
<tr><td>Programmas solis</td><td>Temperatūra</td><td>Laiks</td><td></td></tr>
<tr><td>Sākotnējā denaturācija un polimerāzes aktivēšana</td><td>95 °C</td><td>10 minūtes</td><td></td></tr>
<tr><td>DNS denaturācija</td><td>92 °C</td><td>15 sekundes</td><td rowspan="2">40 cikli</td></tr>
<tr><td>Praimeru hibridizācija un DNS sintēze</td><td>60 °C</td><td>60 sekundes</td></tr>
</table>

Central medicine ethical permission

Centrālā medicīnas ētikas komiteja

Brīvības iela 72, Rīga, LV-1011 • Tālr. 67876182 • Fakss 67876071 • E-pasts: vm@vm.gov.lv

Rīgā

21.02.2018. Nr.3/18-02-21

Latvijas Universitātes Medicīnas fakultātei

Atzinums par pētījumu
"Unificēta terapeitiskā zāļu uzraudzības modeļa izveide pacientiem ar iekaisīgām zarnu slimībām, pielietojot imunoloģiskās, molekulārās bioloģijas un morfoloģijas metodes"

Centrālā medicīnas ētikas komiteja 2018.gada 18.janvārī ir izskatījusi Latvijas Universitātes Medicīnas fakultātes iesniegto pētījumu *"Unificēta terapeitiskā zāļu uzraudzības modeļa izveide pacientiem ar iekaisīgām zarnu slimībām, pielietojot imunoloģiskās, molekulārās bioloģijas un morfoloģijas metodes"*.

Pamatojoties uz Centrālās medicīnas ētikas komitejas 2018.gada 18.janvāra sēdes protokola Nr.2018-1 punktu Nr.3 un iesniegtajiem labojumiem, tiek izsniegts atzinums, ka Latvijas Universitātes Medicīnas fakultātes iesniegtais pētījums *"Unificēta terapeitiskā zāļu uzraudzības modeļa izveide pacientiem ar iekaisīgām zarnu slimībām, pielietojot imunoloģiskās, molekulārās bioloģijas un morfoloģijas metodes"* nav pretrunā ar bioētikas normām.

Centrālās medicīnas ētikas komitejas loceklis

E.Strautiņš

Strautiņš, 67876190
Edgars.Strautins@vm.gov.lv

Genome research council permission

Genoma izpētes padome

Rātsupītes iela 1 k-1, Rīga, LV-1067 • Tālr. 67473083 • E-pasts: genoma.padome@biomed.lu.lv

Rīgā

15.03.2019. Nr.A-5/19-03-15

Latvijas Universitātes,
Medicīnas fakultātes,
Doktorantei Polīnai Zaļizko,

Atzinums par pētījumu: *"Unificēta terapeitiskā zāļu uzraudzības modeļa izveide pacientiem ar iekaisīgām zarnu slimībām, pielietojot imunoloģiskās, molekulārās bioloģijas un morfoloģiskās metodes "*

Genoma izpētes padome izskatīja Latvijas Universitātes, Medicīnas Fakultātes doktorantes Polīnas Zaļizko iesniegumu par pētījumu *"Unificēta terapeitiskā zāļu uzraudzības modeļa izveide pacientiem ar iekaisīgām zarnu slimībām, pielietojot imunoloģiskās, molekulārās bioloģijas un morfoloģiskās metodes"*.

Pamatojoties uz Genoma izpētes padomes locekļu balsojumu, tiek izsniegts atzinums, ka Genoma izpētes padome atbalsta Latvijas Universitātes, Medicīnas Fakultātes doktorantes Polīnas Zaļizko pētījuma *"Unificēta terapeitiskā zāļu uzraudzības modeļa izveide pacientiem ar iekaisīgām zarnu slimībām, pielietojot imunoloģiskās, molekulārās bioloģijas un morfoloģiskās metodes"* īstenošanu.

Genoma izpētes padomes
Priekšsēdētāja vietniece

Z.Daneberga

Rovite, 67473083
vita.rovite@biomed.lu.lv

Pauls Stradiņš Clinical University Hospital Ethical permission

Paula Stradiņa klīniskās universitātes slimnīcas
Attīstības biedrības
KLĪNISKĀS IZPĒTES ĒTIKAS KOMITEJA

Darbojas saskaņā ar SHK LKP un vietējām normatīvajām prasībām
ATZINUMS Nr. 210617 – 7L

1. **Protokola nosaukums: Jauna terapeitiskās zāļu uzraudzības modeļa izveide pacientiem ar iekaisīgām zarnu slimībām.**

2. **Pētījuma protokola numurs: n/a**

3. **Atbildīgais pētnieks un pētījuma centra adrese:**
   Dr. Aldis Puķītis – Paula Stradiņa Klīniskā universitātes slimnīca, Gastroenteroloģijas, hepatoloģijas un uztura terapijas centrs, Pilsoņu iela 13, Rīga, LV-1002, Latvija.

4. **Izskatītie un apstiprinātie dokumenti:**

4.1. Pētnieku CV
4.2. Pētījuma pieteikums ar protokolu
4.3. Pētījuma informācijas lapa latviešu un krievu valodās
4.4. Pacienta piekrišanas lapa latviešu un krievu valodās.

5. **Ētikas komitejas atzinums** – *pozitīvs*

6. **Ētikas komitejas locekļi, kuri piedalījās balsošanā:**

Ilze Aizsilniece – ģimenes ārsts
Dainis Krieviņš – asinsvadu ķirurgs
Biruta Kupča – psihiatrs
Santa Purviņa – farmakologs
Juris Pokrotnieks – gastroenterologs
Inga Vīgante – filologs
Pēteris Ersts – jurists
Daina Biseniece – ķīmiķe

7. **Ētikas komitejas datums: 2017. gada 21. jūnijs.**

Ētikas komitejas priekšsēdētājs **Pēteris Stradiņš**

Paula Stradiņa klīniskās universitātes slimnīcas Attīstības biedrība
Pilsoņu 13, Rīga, LV- 1002, Tel. +371 26380055 fakss +371 67069946; E – pasts: etikas-komiteja@stradini.lv