Inflammatory bowel disease (IBD) encompasses amongst others Crohn’s disease (CD), ulcerative colitis (UC) and IBD unclassified (IBD-u). In the diagnostic algorithm of IBD, ileocolonoscopy plays a pivotal role. [1, 2] Endoscopically obtained histological biopsies are a strong lead in establishing the diagnosis of IBD and to distinguish between the two main phenotypes of IBD; CD and UC. Whereas the worldwide prevalence of IBD is rising and ileocolonoscopy is a relatively invasive and expensive procedure, associated with patient’s discomfort and (severe) complications, researchers have been searching for non-invasive biomarkers to adjust the a priori probability of IBD and to use during the follow-up of disease, to make early diagnosis of flares possible. [3, 4] Up until now, calprotectin (consisting of the protein complex S100A8/S100A9) is the most frequently used biomarker used to make the diagnosis of IBD more or less likely in patients with gastrointestinal symptoms, in which the presentation is not typical for IBD and is used in patients with an established diagnosis of IBD to predict the presence of a flare-up of disease. [5] Whereas the correlation of calprotectin measured in feces with clinically active disease is strong, especially in patients with UC, up to 60% of the patients do not provide fecal samples when asked to, predominantly due to factors as embarrassment or discomfort. [6, 7] However, whereas high concentrations of fecal calprotectin are to date the most specific marker for active inflammatory disease, a low concentration of fecal calprotectin does not rule out active IBD, specifically when proximally located. [5] Furthermore, high concentrations of fecal calprotectin might be present in patients with gastrointestinal infection, ischemia or colonic cancer. [8] For these reasons, part I of this thesis describes the search for a (novel), non-invasive specific biomarker, assessable in peripheral blood, thus making it possible that IBD can be made unlikely without the need of an invasive endoscopic procedure.
Part I: Biomarkers of Inflammatory Bowel Disease

In Chapter 2, the available literature regarding the use of S100A12 as a biomarker of a broad spectrum of diseases was reviewed. S100A12 (also known as calgranulin C) is a calcium-binding protein with inflammatory potentials, secreted by neutrophils. S100A12 is one of the members of the S100 family, of which the combination S100A8/A9 (calprotectin) is mostly used in the field of IBD.

When S100A12 concentrations have to be measured, blood should be drawn in serum tubes for the most representative results, or, if unavailable, heparin tubes may be used. (9) In Chapter 3, a warning is provided for the determination of S100A12 concentrations in EDTA plasma, as these concentrations are not comparable to standardized serum concentrations. S100A12 works by the ligation with the receptor for advanced glycation end-products (RAGE), leading to the production of pro-inflammatory cytokines and increase expression of adhesion molecules. Consequently, pro-inflammatory effects on especially neutrophils and lymphocytes take place. (10) Soluble Rage (sRAGE) is a receptor protein with the same ligand-binding specificity as RAGE, however it lacks cytosolic and transmembrane domains, thus unable to initiate a pro-inflammatory response. By effect, sRAGE might act as a decoy by binding S100A12, withholding them from binding to membrane-bound RAGE.

In Chapter 4, the association of sRAGE with disease severity was described in detail for patients with IBD. It was shown that, especially in patients suffering from UC, a lower concentration of sRAGE was associated to a higher endoscopic severity score. Taken together, despite positive study results from several (S100) biomarkers, ileocolonoscopy remains the cornerstone of the diagnosis and follow-up of patients with IBD.

Once the diagnosis of IBD is established, patients might either have active (relapsing) or quiescent disease (remission). When patients have active disease, induction therapy is needed to get these patients into remission. Once remission is achieved, immunosuppressive therapy is often needed to maintain this state of quiescent disease. [11, 12]
During the induction phase of IBD-treatment, the key drugs are corticosteroids, biologicals and mesalazines (in UC). [11, 12] In patients with either severe disease or poor response to corticosteroid monotherapy, immunomodulatory therapy can be initiated during induction therapy. The addition of biological therapy (e.g. infliximab, adalimumab, golimumab, vedolizumab or ustekinumab) is beneficial in achieving clinical and endoscopical remission. [13] The use of thiopurines as a mono-therapy is not associated with the induction of clinical remission as such, but plays an important role in assuring corticosteroid-free clinical remission in patients with moderate to severe disease. [14-17] Whereas over 80% of the patients experience a relapse within five years after diagnosis, there often is a need for continuous immunomodulatory treatment. [18]

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**Figure 1** Simplified scheme of thiopurine metabolism.

AZA: azathioprine; 6-MP: (6-)mercaptopurine; 6-TG: (6-)thioguanine; 6-MMP: 6-methylmercaptopurine; 6-TUA: 6-thiouric acid; 6-MMPR: 6-methylmercaptopurine ribonucleotides; 6-TIMP: 6-thioinosine monophosphate; 6-TIDP: 6-thioinosine diphosphate; 6-TITP: 6-thioinosine triphosphate; 6-TXMP: 6-thioxanthosine monophosphate; 6-TGMP: 6-thioguanine monophosphate; 6-TGTP: 6-thioguanine diphosphate; 6-TGTP: 6-thioguanine triphosphate; 6-MTG: 6-methylthioguanine

**Enzymatic conversions:**
- GST: glutathione S-transferase
- TPMT: thiopurine S-methyl transferase
- XO: xanthine oxidase
- HGPRT: hypoxanthine-guanine phosphoribosyl transferase
- IMPDH: inosine monophosphate dehydrogenase
- GMPS: guanosine monophosphate synthetase
- ITPase: inosine triphosphate pyrophosphohydrolase
- NUDT15: nucleoside diphosphate-linked moiety X motif 15

Thiopurines are antimetabolites and immunosuppressants firstly described in the 1950s by Gertrude Elion and George Hitchings. [19] Originally, thiopurines were developed for the treatment of childhood leukemia, but due to the immunosuppressive effects as an unwanted side-effect, it was used for the prevention of post-transplant organ rejection later on. In 1962, the first report on the use of mercaptopurine (MP) in the treatment of UC was described by Dr. Bean. [20] From that moment on, thiopurines were increasingly being prescribed as an immunomodulating agent in the treatment of IBD.

Thiopurines are available in the (conventional) derivatives azathioprine (AZA) and MP, and in the more unconventional derivative thioguanine (TG). These conventional thiopurine derivatives are converted into mainly two pharmacologically active metabolites following a multi-enzymatic pathway. In Fig. 1, this pathway is schematically visualized. Azathioprine is converted into 6-MP by the enzymatic activity of glutathione-S-transferase and 6-MP is subsequently competitively metabolized by xanthine oxidase (converting 6-MP into thiouric acid), thiopurine methyltransferase (converting 6-MP into the pharmacologically active metabolite 6-methylmercaptopurine (6-MMP)) or by hypoxanthine-guanine phosphoribosyl transferase into the purine salvage pathway. This pathway leads to the conversion of 6-MP into the pharmacologically active metabolites 6-thioguaninenucleotides (6-TGN, the collective term of 6-thioguanine monophosphate (6-TGMP), 6-thioguanine diphosphate (6-TGDP) and 6-thioguanine triphosphate (6-TGTP)) by the sequential enzymatic activity of hypoxanthine-guanine phosphoribosyl transferase, inosine monophosphate dehydrogenase and guanosine monophosphate synthetase. Thioguanine, a less frequently used thiopurine derivative, is directly converted into 6-TGN by the use of only hypoxanthine-guanine phosphoribosyl transferase. [21] The mode of action in IBD is mainly based on the inhibiting effect of 6-TGTP on the small GTP-ase Rac1, thus inhibiting the anti-apoptotic effect and leading to the apoptosis of CD-28 co-stimulated T-lymphocytes. [22, 23]
Over the past decade, there has been a rising interest in the optimization of (conventional) thiopurine therapy, especially in the patients with an aberrant (so-called skewed) thiopurine metabolism (Table 1). Therapeutic drug monitoring (TDM), thus assessing the concentrations of 6-MMP and 6-TGN in red blood cells of a patient, and the subsequent dose adjustment, co-administration of allopurinol or the switch to another thiopurine derivative are the most practiced optimization strategies. [26, 34, 39] In part II of this thesis, optimization of thiopurine therapy is further evaluated, to warrant safe and effective therapy in IBD patients.

**Part II: Optimizing thiopurine therapy in IBD**

In Chapter 5, we provide a practical overview of the (contra-)indications, start- and stop criteria, toxicity, optimization, cancer risk and teratogenicity of thiopurine therapy in IBD patients. In this clinical review, recommendations to guide dosing in individual situations are provided to (IBD-treating) gastroenterologists. More specific, all possible scenarios of thiopurine metabolite measurements were given, providing useful suggestions for each situation, varying from patient motivation to gain compliance, drug dose enhancement, considering allopurinol co-administration or switch thiopurine therapy to TG. This last suggestion has been related to the development of nodular regenerative hyperplasia (NRH) of the liver from the beginning of this century, possibly leading to non-cirrhotic portal hypertension. [40] From that moment onwards, it was demonstrated in multiple studies that NRH seems to be a dose-dependent adverse event of TG therapy, and that the occurrence in IBD patients treated with low-dose TG was similar to the occurrence in the thiopurine-naïve population. [41, 42]

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TPMT: thiopurine-S-methyltransferase; N/A: not applicable

Table 1 Overview of thiopurine optimization strategies.
Furthermore, the presence of histological NRH was not associated with clinical significant liver disease in the majority of IBD-patients treated with TG. [43]

To bring the results of this review into perspective, we showed the results of our real-life 10-year cohort of patients with IBD treated with thiopurines in Chapter 6. Whereas the effects of TDM and allopurinol co-administration have been assessed in several clinical trials, evidence in a real-life setting without strict monitoring procedures was lacking. [34, 44] In this largest real-life cohort to date, we showed the beneficial effects of allopurinol co-administration after TDM, mainly in preventing (hepatotoxic) adverse events, which underlined earlier clinical trials examining this drug combination. [34, 36, 45] Furthermore, clinical benefit as a secondary outcome, was reported in about 40% of the patients included in the most recent cohort, which was over two-fold higher than the first cohort, showing that optimization of thiopurine therapy improves the use of conventional immunosuppressants in IBD-patients. [46, 47]

In Chapter 7, the effect of thiopurine metabolites on hematologic parameters was assessed. 6-Thioguaninenucleotides are structurally similar to the endogenous purine bases, for which reason these may be incorporated into DNA or RNA as fraudulent bases, resulting in an anti-metabolic effect by causing strand breakage and subsequently cytotoxicity, but only when high concentrations of 6-TGN are present. [48, 49] On the other hand, 6-methylmercaptopurines can occasionally (in high concentrations) cause leukopenia by inhibition of the de novo purine synthesis, which inhibits the generation of DNA. [50] We objectified that mainly the white blood cell count was negatively associated with AZA and MP therapy, but this association was not reproduced in the patients receiving TG, despite higher 6-TGN concentrations in this group. One of the possible explanations for this finding is the fact that 6-TGN are measured in red blood cells, whereas the immunosuppressive effect mainly takes place in white blood cells (WBC). This is derived from the original indication of thiopurines, being the treatment of (childhood) leukemia. As a consequence, measurement of metabolites is performed in red blood cells,
since WBC are unavailable in successful leukemia treatment. [19] In this chapter, to correlate 6-TGN concentrations in WBC, we calculated the measured 6-TGN concentrations by a conversion method as suggested in the paper by Lancaster et al. [51] and we demonstrated a negative correlation between 6-TGN in WBC and leukocyte count.

In Chapter 8, we demonstrated that up to 50% of the IBD patients intolerant or refractory to AZA therapy benefitted from a switch to MP, the converted compound of the pro-drug AZA, on itself available as off-label therapy for treating IBD. As earlier observed, a substantial proportion of IBD patients with intolerance for AZA was able to tolerate MP therapy, despite the fact that the pharmacologically active metabolites are similar (6-MMP and 6-TGN). [31, 52] One of the reasons for this difference might be the pharmacokinetic profiles of the two drugs, as AZA is a pro-drug for 6-MP, bypassing the first-pass mechanism observed in patients taking MP. [25] Half of the included patients in Chapter 8 were relatively under-dosed (i.e. AZA < 2.0 mg/kg or MP < 1.0 mg/kg), for which reason it is doubtful what the effect of this different pharmacokinetic profile is on the outcome in this group. Further, as the imidazole group is split off during conversion of AZA into MP, this molecule might instigate adverse events in addition. [30] Very recently, in a pooled sub-analysis of the TOPIC trial, a prospective cohort study initiated to determine cost-effectiveness of TPMT genotyping in IBD patients before the start of thiopurine therapy, it was observed that patients with MP therapy were more likely to develop (dose-dependent) adverse events, as these patients were having higher 6-MMP and 6-TGN concentrations, potentially due to higher dosing (mg/kg) as compared to the AZA group (adjusted AZA dosage 1.05 vs. MP dosage 1.21 mg/kg, P < 0.001). Interestingly, this difference in experienced number of adverse events did not cause higher discontinuation rates (within 5 months) between the groups (AZA: 39.3% vs. MP: 38.1%, P = 0.50). [53]
Chapter 9 consisted of a systematic review of the available literature regarding the effectiveness of TG in IBD patients. Where researchers have mainly focused on the safety profile of TG, relatively little is known about the effectiveness of this drug. In this chapter, we demonstrated that TG is an effective alternative in about 65% of the patients intolerant to conventional thiopurine therapy. Short-term adverse events of TG were observed in one fifth of the patients, which comprised mainly aspecific adverse events, such as gastrointestinal complaints, hypersensitivity reactions and elevated liver enzymes.

To assess the effectiveness and tolerability of TG as a rescue drug in UC, we compared it with methotrexate (MTX), another immunosuppressant used as rescue therapy in UC. For this comparison, we scrutinized the Parelsnoer database, a national database initiated by the Netherlands Federation of University medical centers (NFU), and depicted the results in Chapter 10. We demonstrated favorable short- and long-term tolerability results, with 70% of the patients in both drug groups able to continue treatment for at least one year, a percentage similar to that observed in patients receiving conventional thiopurine derivatives. [12, 54, 55] Subsequently, almost half of the patients able to tolerate TG/MTX therapy experienced clinical benefit (i.e. the ability to continue therapy without the need of intensified treatment or surgery) of this therapy.

In the search for novel therapeutic drug monitoring options to optimize treatment with thiopurines in IBD, we assessed the inter-individual variability of xanthine oxidase (XO) in Chapter 11. We demonstrated a gender-specific distribution of XO (higher activity in males vs. females) and a positive correlation of XO-activity with age. This study was the first to measure XO-activity in IBD patients and might form a startpoint for future correlation studies with XO-activity, thiopurine dosage and metabolite concentrations.
As any drug, thiopurines have been associated with the development of several dose-dependent or compound-related (idiosyncratic) adverse events. Two of the most feared adverse events are myelotoxicity (depression of red and white cell lines, as well as platelet generation) and hepatotoxicity (mostly presenting as a rise in alkaline aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations). The development of myelotoxicity has been related to high 6-TGN concentrations, whereas hepatotoxicity has predominantly been related to high concentrations of methylated products (6-MMP). [25, 56, 57] In part III of this thesis, the pathogenesis and prevalence of these adverse events during thiopurine therapy are described in more detail.

**Part III: Complications of thiopurine therapy**

Despite the fact that myelotoxicity is related to 6-TGN in medical literature, myelotoxicity (i.e. leukopenia) may also occur in patients with (extremely) high 6-MMP concentrations, as illustrated in Chapter 12. When 6-MMP concentrations get (extremely) high, this can cause inhibition of the de novo purine synthesis, thus causing cytotoxicity. [50] This situation occurs in patients with a skewed thiopurine metabolism, and in Chapter 12, we demonstrated that almost all patients were able to continue thiopurine therapy with an adapted regimen (i.e. dose reduction, allopurinol co-administration or switch to TG).

In the past, a direct relationship between thiopurine-use and the development of NRH has been described. [40] In a small proportion of patients, NRH led to the development of severe portal hypertension. To assess the incidence of NRH-based end-stage liver disease warranting liver transplantation, we describe the results of our nation-wide database study comprising all liver transplant units from The Netherlands in Chapter 13. Whereas the association of NRH with severe complications of portal hypertension is not well established in patients with IBD [43], we scrutinized this database over the past 20 years. In total, 11 patients underwent liver transplantation due to NRH, with various causal factors. In five of these patients, thiopurines were used at some point during the underlying disease course, however in three of these five patients, other factors knowing to
cause NRH (e.g. hematologic malignancies or chemotherapeutic treatment) were simultaneously present, dissembling the causal role for thiopurines in these patients. These results are in line with recently published papers on the clinical disease course of NRH caused by thiopurines in IBD patients, showing that the majority of IBD-patients with thiopurine-induced NRH do not experience (severe) symptoms of portal hypertension. [43]

To optimize safe therapy in IBD patients, detection of (early) toxicity plays a pivotal role. As the prevalence of histologically assessed hepatic fibrosis amongst IBD-patients was common in earlier performed studies, regardless of thiopurine exposure, we assessed the value of non-invasive methods to diagnose fibrosis in an early stage. [42] In Chapter 14, we describe our results of 200 consecutive IBD-patients who underwent transient elastography measurements using Fibroscan. We observed higher mean liver stiffness in males vs. females and in patients not exposed to thiopurines compared to patients with (either historical or current) thiopurine exposure. Whereas the number of patients with moderate to severe fibrosis in our study population was too small to draw firm conclusions, we believe this study is a stepping stone for future studies, assessing the correlation between transient elastography measurements and histopathological biopsies.
Conclusion
In conclusion, novel insights in the use of non-invasive (serum) biomarkers to guide the diagnosis and follow-up, and to predict the severity of disease in patients with IBD were provided in the first part of this thesis. Biomarkers can, at this moment, not outclass findings of ileocolonoscopy.
In the second part of this thesis, we provided strategies to optimize safety and effectiveness of thiopurine therapy in IBD patients by the use of allopurinol as co-therapy or the use of thioguanine as an unconventional thiopurine derivative.
Finally, we described the pathogenesis and management of two adverse events of thiopurine therapy (myelotoxicity and hepatotoxicity) in detail and provide new viewpoints on the safety of thiopurines as a treatment in IBD. In this thesis, clinical guidelines for the optimal use of thiopurines were provided.
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