

METHODOLOGY

To achieve the aim of the study, up-to-date analytical methods will be used and for drugs where current procedures are not readily available these will be developed. For a methodical effectivization (aimed at an easier practical/clinical/personal implementation), and as well as environmental benefits, simultaneously with reference analytical methods and procedures alternative analytical approaches (beyond the currently available methodical solutions) will be developed and evaluated as an integral part of the project. Depending on the size of the datasets, proper statistical methods will be applied. During the duration of the project, all patients treated in two referral IBD centra with JAKi will be included and the samples will be taken at prespecified time points (Table 1).

This approach is complex and has not been used in the various attempts to elucidate the mechanisms of treatment failure in immune suppressive treatment of IBD so far. The continuation of the project beyond the currently described frame will bring new insight into the mechanism of response to respective drugs used in the treatment of IBD and will thus allow for individualized treatment in the future (expected benefits beyond the state of the art).

Table 1. List of samples obtained for JAKi pharmacokinetics analysis

Drug	Timepoints of the treatment	Sample type
Tofacitinib	- prior treatment - levels at week 8 of induction therapy - every 2 months of maintenance therapy	- serum - urine - intestinal biopsy*
Upadacitinib	- prior treatment - levels at week 8 of induction therapy - every 2 months of maintenance therapy	- serum - urine - intestinal biopsy*
Filgotinib	- prior treatment - levels at week 10 of induction therapy - every 2 months of maintenance therapy	- serum - urine - intestinal biopsy*

*at selected time points when endoscopy will be planned for clinical follow-up

For each drug, between 20 to 30 patients will be included. All patients willing to participate by the means of giving informed consent will be eligible. The patients will be followed for a minimal period of 6 months, and long-term follow-up (2 years) will be subject to a continuation of the study beyond the time scope of this project. Patients' demographics, disease phenotype, and behaviour as well as responses to previous therapies will be noted. The response to treatment will be evaluated clinically (Mayo score or Harvey Bradshaw Index), biologically (normalization or decrease of C-reactive protein, normalization of increase of haemoglobin), by ultrasound, and endoscopy. The levels of respective drugs will be assessed at time points shown in Table 1 and the differences between responders and non-responders will be analysed statistically for each particular body compartment.

Concerning analytical methodology for JAKi, they are of low molecular weight compounds, therefore their analysis will be performed principally in a similar way to other low molecular IBD drugs. Reference LC-MS approaches, dominating for JAKi applications in literature, must be optimized concerning the specific character of JAKi molecules and accompanied sample matrices. Considering the different polarities of thiopurines metabolites on one side and JAKi on the other side, for the former (highly polar) analytes IEC or HILIC chromatography modes are naturally preferred (see works, e.g., (Hofmann et al., 2012; Keuzenkamp-Jansen et al., 1995)) while for the later (relatively less polar) analytes is preferred RP (C8, C18) chromatography mode (see current works, e.g., (Dixit et al., 2020; Kim et al., 2020; Koller et al., 2020; Sharma et al., 2015)). In the case of PGC chromatography, proposed for thiopurines in our recent work (see thiopurine section), there is some overlap with standard RP phases and some degree of graphitization gives the material other distinctive properties. It enables PGC to provide retention for analytes possessing a wide scale of polarities. Hence, the development of novel reference LC-MS methods, considering PGC-MS as a potentially attractive alternative to the published RPLC-MS methods for the determination of JAKi in biological systems related to IBD, is the main goal of this part of the project.

This will be methodically performed in two pathways:

1. Development of reference LC-MS methods for determination of JAKi levels in biological matrices, comparative studies (RP vs. PGC, HILIC). Study of 1D and 2D column approaches.
2. Development of analytical methods alternative to a reference LC-MS approach (or its 2D counterpart), for selected analytes/applications in the scope of the presented project – innovative electromigration and electrochemical methods for JAKi.

Besides the reference methods, namely, high-performance liquid chromatography (or supercritical fluid chromatography for thermally less stable analytes, e.g., thiopurine nucleotides) hyphenated with tandem mass spectrometry (QQQ, QTOF), alternative methods for specific purposes will also be developed. Electromigration (capillary electrophoresis, isotachopheresis, electrokinetic chromatography) and electrochemical (CV, SWV, DPV, etc.) techniques will be employed to develop such alternative analytical methods for selected analytes (charged, electrochemically active), for which enhanced performance parameters (analysis time, selectivity, sensitivity, etc.) can be easily obtained along with additional benefits laying mainly in cost and simplicity of instrumentation/measurement and green analysis protocols. Analytical procedures and interconnectedness of particular stages during their development are following: (i) sample preparation for given analyte (drug) and sample matrix (Table 1) and used analytical method, (ii) method optimization and innovation (including implementation of innovative issues into analytical technologies such as new chromatographic stationary phases, electrochemical sensors and modifiers, newly designed analytical agents (also from our own portfolio, i.e., triazine derivatives), two dimensional systems (SPE-UHPLC-MS/MS, SFC/HPLC-MS/MS, ITP-CZE-MS/MS, ITP-EKC-MS/MS); this stage is carried out usually in two steps: (iia) method optimization in model matrices/samples (not requiring ethical commission statement), (iib) method optimization in real matrices/samples (patients, requiring ethical commission statement), (iii) method validation (ICH, FDA, EMA protocols), (iv) comparative study with two independent methods, (v) proposal of validated methodical protocol (whole procedure of sample handling and storage, operational and validation parameters of analytical method) – ready to be disseminated and implemented into gastroenterological research as well as clinical practice (control clinical labs), (vi) method application (determination of analytes levels in biological systems (patients' samples), Table 1, pharmacokinetic studies, therapeutic drug monitoring), (vi) statistic evaluation of analytical data, testing of hypothesis (from t-test to multivariate ANOVA, depending on character and size of analysed datasets and demanded information) to indicate relationships and statistical significance of parameters of interest (here within patient's characteristics vs. therapy specifications vs. analytical profile vs. clinical response/non-response). Prospectively, depending on the degree of sample database building, for the large well-structured datasets, point (vi) will be completed by the proposal of highly reliable multivariate models for the predictions of particular clinical responses, with subsequent implementation of such models to the clinical practice for IBD therapy individualization.

References:

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