MDR—ABC transporter activity in normal population and in rheumatoid arthritis patients.
A predictive tool of biological therapeutic response.

Ph.D Thesis

Péter Gábor Szerémy

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Articles related to the subject of the thesis

I. Szerémy P, Tauberné Jakab K, Baráth S, Apjok A, Filkor K, Holló Z, Márki- Zay J, Kappelmayer J, Sipka S, Krajcsi P, Toldi G. Determination of Reference Values of MDR-ABC Transporter Activities in CD3+ Lymphocytes of Healthy Volunteers Using a Flow Cytometry Based Method. Cytometry B Clin Cytom. 2019 Nov;96(6):469-474. doi: 10.1002/cyto.b.21729.

II. Toldi G, Batel P, Baráth S, Szerémy P, Apjok A, Filkor K, Szántó S, Szűcs G, Szamosi S, Häupl T, Grützkau A, Szekanecz Z. Peripheral Lymphocyte Multidrug Resistance Activity as a Predictive Tool of Biological Therapeutic Response in Rheumatoid Arthritis. J Rheumatol. 2019 Jun;46(6):572-578. doi: 10.3899/jrheum.180793.

Patent related to the subject of the thesis:

III. Toldi G, Filkor K, Szerémy P, Apjok A, Szekanecz Z Assessing Responsiveness of Rheumatoid Arthritis Patients to Biological Treatment. Patent No: EP3570028A1
Priority Data: 15.05.2018

Table of Contents

List of abbreviations

ABC	ATP-binding cassette
ACPA	anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ADME	absorption, distribution, metabolism and excretion
anti-CCP	anti-cyclic citrullinated peptide
AUC	area under the curve
BBB	blood–brain barrier
BCRP	breast cancer resistance protein
bDMARD	biological disease modifying anti rheumatic drug
calcein-AM	calcein acetoxy methyl ester
CD	cluster of differentiation
CDAI	clinical disease activity index
CLSI	Clinical Laboratory Standards Institute (former NCCLS)
CLSI	Clinical and Laboratory Standards Institute
CRO	contract research organization
CRP	C-reactive protein
csDMARD	conventional syntheticdisease modifying anti rheumatic drug
CV	coefficient variation
DAS28	disease activity score 28 joint count
DMARD	disease-modifying anti-rheumatic drug
DNA	deoxyribonucleic acid
EULAR	European League Against Rheumatism
FITC	fluorescein isothiocyanate
FLS	fibroblast-like synoviocytes
FSC	forward scatter characteristics,
g	gravitational force equivalent
HBSS	Hanks' Balanced Salt Solution
IFN	interferon
IL	interleukin
IQR	interquartile range
IVD	In vitro diagnostic

K3EDTA	ethylenediaminetetraacetic acid tripotassium
LDA	low disease activity
MAF	MDR activity factor
MAFC	composite MAF of MRP1 and MDR1
MDR	multidrug resistance
MDR1	multidrug resistance protein 1
MFI	mean fluorescent intensities
mRNA	messenger RNA
MRP1	multidrug resistance-associated protein 1
n	sample size
NF-κB	nuclear factor-κB
NICE	National Institute for Health and Clinical Excellence
PAF	platelet-activating factor
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PET	positron emission tomography
PhD	philosophiae doctor
RA	rheumatoid arthritis
RF	rheumatoid factor
ROC	receiver operating characteristic
S1P	sphingosine-1-phosphate
SDAI	simple disease activity index
siMDR1	small interfering RNA for MDR1
SLC	solute carrier
SSC	side scatter characteristics
Th	T helper cell
TM	trademark
TNF	tumor necrosis factor

1. Introduction

In the past 10 years, a paradigm shift has taken place in understanding the clinical significance of mechanism of action of multidrug resistance (MDR) transporters and their function as potential biomarkers.

Earlier, it was assumed that the mechanism behind impaired therapeutic efficacy of certain drugs and MDR—ABC transporter activity is solely associated with the drug efflux function. In addition to drug transport more and more evidence emerged that the transporters have an important role as regulators of inflammation and immunity mediators e.g. lipids and redox regulators. Thus, in autoimmunity and also in tumor biology MDR—ABC transporters might play a role in the immune regulation representing the most important efflux mechanism for several inflammatory signaling molecules.

According to the new concept MDR—ABC transporters are biomarkers. Their role in the immune processes and MDR can only be evaluated as part of a complex panel of biomarkers for prognostic scoring, for monitoring disease activity or to predict the responsiveness to certain medications (e.g. immunosuppressive treatments or chemotherapy in malignancies) However, translation of MDR—ABC transporter activity into clinical decisions and treatment regimen requires well defined normal reference and pathological activity values.

This thesis explores how the new scientific findings might establish MDR—ABC transporters as predictive biomarkers in rheumatoid arthritis (RA).

1.1. Transporter nomenclature

In general ATP-binding cassette (ABC) transporters have one systemic name and more traditional names.

ABCB1 as multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp)

ABCC1 as multidrug resistance-associated protein 1 (MRP1)

ABCG2 as breast cancer resistance protein (BCRP) or mitoxantrone resistance protein (MXR-1)

For the purpose of this thesis I am using the same nomenclature (MDR1 / MRP1 / BCRP) as in the papers listed in the “Articles related to the subject of the thesis” section.

1.2. Different approaches in MDR—ABC transporter research.

There are many different approaches applied in today's transporter science to study the role of transporter mediated MDR and immune modulation. Mrembramnetransporters comprise two superfamilies the ABC transporter superfamily that mostly mediate drug efflux and the solute carrier (SLC) superfamily that mostly mediate drug influx. Although the SLC supetfamily is large and very significant from both a pharmaceutical and a diagnostics point of view this thesis will focus on MDR—ABC transporters.

I. Role of transporters in disease

   Transporters and transporter substrates as biomarkers in a novel diagnostic approach.

   As this application is the main topic of the thesis detailed discussion is presented. Focused on the importance of the MDR1, MRP1 and BCRP transporters in RA [1,2].

II. Transporters as therapeutic targets

   Early applications focused on development of MDR reversing agents to improve cancer therapy. This is still an actively researched area with potential applications in other diseases. A novel direction of this field aims to exploit collateral sensitivity of cancer cells overexpressing MDR—ABC transporters. This is, however, a new field with aspects that may point to non-transporter related mechanisms [3].

III. Transporters as mediators of drug ADME / pharmacokinetics

   MDR—ABC transporters such as MDR1 and BCRP play significant role in absorption, distribution, metabolism and excretion (ADME) / pharmacokinetics of substrate drugs by limiting absorption, distribution and mediating excretion. Therefore, this is a highly researched and applied aspect in drug discovery and development.

   The role of ABC proteins in resistance to drugs has been known for over 50 years. Cancer multidrug resistance is the most studied and defined as the cross-resistance or insensitivity of cancer cells to the cytostatic or cytotoxic actions of various anticancer drugs which are structurally or functionally unrelated and have different molecular targets [4]. A total of 15 family members can function as drug-efflux pumps, and have been implicated in potentially conferring resistance to chemotherapeutic agents. However, MDR1, MRP1, BCRP to account for most observed MDR linked to clinical cases of drug resistance .

IV. Transporters as mediators of drug toxicity and safety

Modulation of transport of physiological substrates with a relevance to toxicity and modulation of transport of co-administered drugs (drug – drug interaction).

MDR—ABC transporters efflux endogenous compounds and inhibition of these transporters may precipitate toxicity.

Inhibition of transport of co-administered drugs may increase plasma or tissue levels of substrate drugs affecting drug safety.

In pharmacokinetic type drug - drug interactions perpetrator drugs modulate plasma or tissue levels of victim drugs. Drug-drug interactions have a tremendous clinical relevance especially if we consider that patients are often administered several drugs simultaneously. Studies confirmed that the cause of an unexpected side effect of a given drug (transporter substrate) can be attributed to the other administered drug (modulator) that is interacting with a drug efflux transporter. This may lead to potentially harmful, even life-threatening side effects underlying the clinical importance of drug efflux transporters in drug administration.[5]

All approaches have clinical implications but the nature and the extent of the implications is diverse and even controversial. This is why despite of huge scientific interest in transporters clinical exploitation of research results is rare.

1.3. The phenomenon of multidrug resistance (MDR)

MDR is a term used to describe the phenomenon whereby cancers become resistant to multiple anti cancer drugs that are chemically not related to each other. Historically the concept of MDR in tumors originated from the pioneer work of June Biedler in the early 1970s, who identified a wide profile of cross - resistance in Chinese hamster cells selected for resistance to actinomycin D. It was shown that a glycoprotein of 170 kiloDalton, called P-glycoprotein, correlated with the degree of drug resistance in several cell lines. A variety of cells were found that were resistant to a number of popular anticancer drugs, such as doxorubicin[6].

P-glycoprotein or MDR1 was purified in 1979, and strong evidence in support of its role in pleiotropic drug resistance came in 1982. It was shown that deoxyribonucleic acid (DNA) from resistant cell lines that was transferred to nonresistant cells was able to confer resistance to the latter that correlated with the expression of the protein [7,8]. The gene for MDR1, was cloned in 1985,

and the protein's putative function as an energy-dependent pump that expels small molecules from inside cells was established [9].

Work on a lung cancer cell line that was resistant to doxorubicin and other chemotherapeutic agents showed that this cell line did not overexpress P-glycoprotein, but did express another protein, namely MRP, cloned in 1992. MRP was also found to be a drug efflux pump, specifically a member of the ATP-binding cassette transmembrane transporter superfamily [10].
Subsequent to the discovery of MRP1, various groups began reporting non - MDR1 -, non - MRP1 - mediated drug resistance in cell lines selected with mitoxantrone. In addition to high levels of mitoxantrone resistance, these mitoxantrone - selected cells often displayed resistance to doxorubicin and etoposide, but not to vinblastine or cisplatin. Doyle and colleagues were the first to clone the transporter, calling it BCRP for breast cancer resistance protein, since it was cloned from a human breast cancer subline. BCRP was characterized as a multidrug resistance efflux transporter in 1998, and appears to be the second member of the "G" subfamily of ABC transporters [11]. Doyle et al. reported high levels of BCRP expression in the placenta, as well as lower levels in the brain, prostate, small intestine, testis, ovary, and liver.

Mass spectrometry-based proteomics studies has shown high level expression of BCRP in enterocytes [12] as well as in blood-brain barrier endothelial cells [13] and lower expression in hepatocytes [14] and kidney proximal tubule cells [15]. In lymphocytes mRNA levels of BCRP was highest in cord blood samples at birth and decreased significantly during the first month of life to an expression level similar to that in adults [16].
ATP -binding cassette proteins i.e. the ABC transporters like MDR1, MRP1 and BCRP are one of the largest transmembrane protein superfamilies encoded in the human genome and are generally responsible for the energy-dependent efflux of xenobiotics, including a wide spectrum of hydrophobic drugs.

1.4. Clinical relevance of MDR—ABC transporters

Although the MDR phenomenon was firstly described in tumour cells, MDR—ABC transporters have been identified in many normal tissues including immune cells as part of a mechanism of the resistance to antiviral [17, 18] and immunosuppressive [19, 20] therapies. MDR—ABC transporters have been linked to transport of a variety of endobiotics and implicated in various processes of cancer development such as proliferation, metastasis, inflammation and stem cell survival [21, 22].

Such endobiotics secreted by the MDR—ABC transporters play important roles in inflammatory response due to the differentiation, proliferation and maturation of immune cells as well as in their migration into the inflamed tissues [23]. MDR1 not only transports hydrophobic and positively charged drugs [24] but also transports, pro-inflamatory molecules like platelet-activating factor (PAF) [25] and various other membrane lipids including cholesterol [26] and sphingolipids [27]. Transport of PAF may facilitate angiogenesis [28] while cholesterol [29] as well as sphingolipids [30] modulate drug resistance. In addition to acidic and hydrophobic drugs MRP1 transports important mediators of inflammation prostaglandins and leukotrienes a family of unsaturated fatty acids derived by metabolism of arachidonic acid, a component of most cell membranes, [15]. MRP1 and BCRP transport sphingosine-1-phosphate (S1P) [31] that facilitates cell growth, survival, invasion and angiogenesis. TNF-α can activate S1P production. Indeed S1P levels are higher in the synovium of RA patients. S1P administration to RA synovial fibroblasts increase proliferation, survival, and migration, as well as cytokine/ chemokine and other proinflammatory mediator production. Suggesting that S1P may play a role in RA pathology [32]. BCRP transports drugs with a wide substrate specificity [33]. It also transports various vitamins, such as folates (folic acid / vitamin B9) [124] and flavins (riboflavin/vitamin B2) [35, 36]. Cellular efflux of folates may aggravate folate deprivation in patients on methotrexate therapy. Therefore, MDR—ABC transporters are “more than just drug efflux pumps” [21]. MDR—ABC transporter interaction of anti-rheumatic drugs is summarized in Table 1. The anti-inflammatory effect of disease-modifying anti-rheumatic drugs (DMARDs) might be at least partially attributable to the inhibition of the pathophysiological function of the MDR—ABC transporters in immune cells [37].

Laboratory and preclinical investigations have revealed that redox signaling is a major stress-response process associated with the treatment of antitumor agents. Evidence has accumulated showing that redox signals are activated in response to drug treatments that affect the expression and activity of MDR—ABC transporters by multiple mechanisms. MDR cells exhibit paradoxical hypersensitivity towards a diverse set of redox agents.[135] In 2021 Pape at.al showed that the MDR-selective anticancer activity of 8-hydroxyquinoline derivatives is associated with the iron deprivation of MDR cells and oxidative stress induction. [55]

Literature data proves that besides as regulators of inflammation and immunity mediators MDR has a tremendous clinical relevance as increased drug efflux is the principal mechanism by which many cancers develop resistance to chemotherapic drugs. Moreover, as chemotherapeutic agents are also widely used to suppress human immune system, MDR may have clinical relevance in systemic inflammatory diseases and in post-transplantation conditions. Recent data suggest that certain

transporter proteins might be involved in the inflammatory pathways, which opens up new exploitation opportunities for new applications in clinical practice.

Therefore, potential clinical implications of MDR are very diverse and in many aspects have not been fully explored. [38]

Transporter	**Selected drug substrates**	**Physiological substrate**	**Experimental substrates**
MDR1	chloroquine [38] prednisone [38] hydrocortisone [39] dexamethasone [39] aldosterone [39] cyclosporin A[40]	PAF [25] lipids [26] sphingolipids [26] steroids [41]	calcein-AM [42] rhodamine 123 [43]
MRP1	chloroquine [38] methotrexate [44],	leukotriene C4 / B4 / E4 [41] prostanoids[41] folates[34]	calcein-AM[42]
BCRP	mitoxantrone [11] methotrexate [45] sulfasalazine [46] leflunomide [47] hydroxychloroquine [48]	steroids, chlorophyll metabolites, urate [41] folates [34] flavins [35]	mitoxantrone [11]

Table 1.*: List of MDR—ABC transporter substrates, have been covered in this thesis.*

There are 49 known ABC transporter gens in the human [49], and there are at least 14 ABC genes linked to specific human genetic disorders. Human genetic disorders usually linked to the transport of physiological substrates or endobiotics. Among the three detailed MDR—ABC transporters BCRP is closely linked to hyperuricemia.[41]. As this aspect is not the main focus of the thesis it will not be discussed in more detail.

1.5. MDR in autoimmune disorders

Research on the MDR1 phenotype in autoimmune disorders, particularly in rheumatoid arthritis (RA), began in the mid-1990s with works from the French teams of Jorgensen et al. [92] and Maillefert et al.[86] They described, respectively, high MDR1 messenger RNA (mRNA) levels in synovium from RA patients with history of treatment with three or more second-line agents, and MDR1 surface overexpression in peripheral lymphocytes from RA patients under long-term steroid therapy. These reports suggest that in RA patients receiving steroids and/or immunosuppressive

drugs [38], MDR1 overactivity in immune cells might be associated with increased efflux of these agents and, consequently, with an insufficient therapeutic effect. Studies investigating the possible role of ABC transporters in autoimmune disorders e.g.: RA [50] and systemic lupus erythematosus [51], immune throbocytopenic purpura [52] and myasthenia gravis [53] concluded that enhanced MDR1 activity may be closely related to an unfavorable clinical course and a poor response to treatment.

Moreover it was confirmed that MDR1 serves several distinct functions in the initiation of primary immune responses, and a critical role of the molecule in functional immune responses [23]. Based on these findings it was hoped that MDR1 would be novel therapeutic target for immune modulation [27] in acute and chronic allograft rejection, and cell-mediated autoimmune disorders. According to the new concept MDR—ABC transporters are biomarkers. Their role in the immune processes and MDR is a rapidly developing field and it will be likely evaluated as part of a complex panel of biomarkers for prognostic scoring, monitoring disease activity or to predict the responsiveness to immunosuppressive treatments.

Research data suggest that the MDR drug transporters might play a role in the immune regulation [35] representing the most important efflux mechanism for several inflammatory signaling molecules, such as eicosanoids (prostanoids and leukotrienes), which are among the mediators of chronic inflammation [21]. MDR—ABC transporters may also modulate cellular redox homeostasis [55, 125]. In addition, several studies have showed the clinical significance of MDR as prognostic and/or predictive marker in immunosuppressive therapies for active RA using methotrexate, other synthetic DMARDs (sDMARDs) or biological DMARDs (bDMARDs). The methods included the measurement of functional activity, gene- or protein expression of MDR—ABC transporters, mainly MDR1.

1.6. Rheumatoid arthritis

Rheumatoid arthritis is one of the most common chronic inflammatory autoimmune diseases affecting about 0.5–1% of the world population.

RA is more prevalent in female and it is often occurred at the 5th decade of the life [56]. The disease is characterized by the overactivation of the immune system and progressive joint destructions [57]. Persistent synovial inflammation finally results in joint and bone malformation [58] which causes disability, that drastically cuts down the patient’s quality of life [59]. As a consequence of

widespread inflammation the function of other organs and tissues such as the heart, the lung and the blood vessels are impaired as well.

In the last 20 years, drastic improvements in RA treatment have been developed by applying a wide variety of new synthetic and biologic DMARDs such as anti-tumour necrosis factor (TNF) antibodies (e.g adalimumab, certolizumab pegol, etanercept) and T-cell inhibitors (e.g abatacept) [57, 60]. Importantly, early intervention with DMARDs maintains long-term functional activity of affected joints, by preventing them from tissue damage [61,62].

The trigger of pathogenesis of RA is still obscure. The pathophysiology of RA involves interactions of innate and adaptive immune systems. Cells participating in pathogenesis are valid cellular targets for small molecule therapy. Interplay of T cells and B cells determines the autoimmune process leading to inflammation and destruction of affected joints. During this process the T helper cells (Th1/Th2) and Th17/Treg balance becomes shifted towards formation of the inflammatory Th1 and autoreactive Th17 cells [63]. These cell subsets then produce various inflammatory cytokines upon interaction with antigen presenting cells. Th1 cells activate B cells to produce auto-antibodies (e.g. rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) [64]. Activated B cells differentiate into plasma cells that produce large quantities of these antibodies. Importance of B cells is substantiated by the therapeutic efficacy of rituximab, the anticluster of differentiation-20 (CD-20) antibody that efficiently deletes B cells [65]. The other downstream cellular targets of the two T cell subset (Th1, Th17) are macrophages in the synovial tissue. Macrophages contribute to abundance of inflammatory cytokine, tumour necrosis factor (TNF) in the synovium [66]. The T cell macrophage interaction is mediated via secreted cytokines interferon (IFN)-gamma and interleukin (IL-17). IL-17 plays a major role in tissue destruction as this cytokine activates fibroblast-like synoviocytes (FLS) and osteoclasts, two effector cell types secreting matrix metalloproteases and invading cartilage [67]. FLS express both IL-15 and IL-15 receptor (IL-15R), therefore they may proliferate in an autocrine manner [68]. Activation of polymorphonuclear leukocytes in RA exacerbates inflammation due to production of prostaglandins and leukotrienes as well as direct tissue damage via released lysosomal enzymes and superoxide anions [69].

The ubiquitously expressed nuclear factor-κB (NF-κB) transcription factor participates in the survival, proliferation, activation and differentiation of pro and anti inflammatory cells. NF-κB activation in Synovial Macrophages and FLSs not only enhances the production of pro-inflammatory cytokines and matrix metalloproteinases, but also promotes proliferation and inhibits apoptosis, which leads to disease progression. [140]

Among other factors CD3+, DC19+, CD69+ cells emerged as potential predictors for response to nonbiologic or biologic disease-modifying antirheumatic drugs. [70] Compared to ACPA- RA

patients, synovium from ACPA+ RA patients was characterized by significantly higher levels of CD19+ B cells and CD3+ and CD8+ T cells (each $P < 0.05$), and CD19+ B cell levels were significantly higher in patients who were naive to treatment [71].

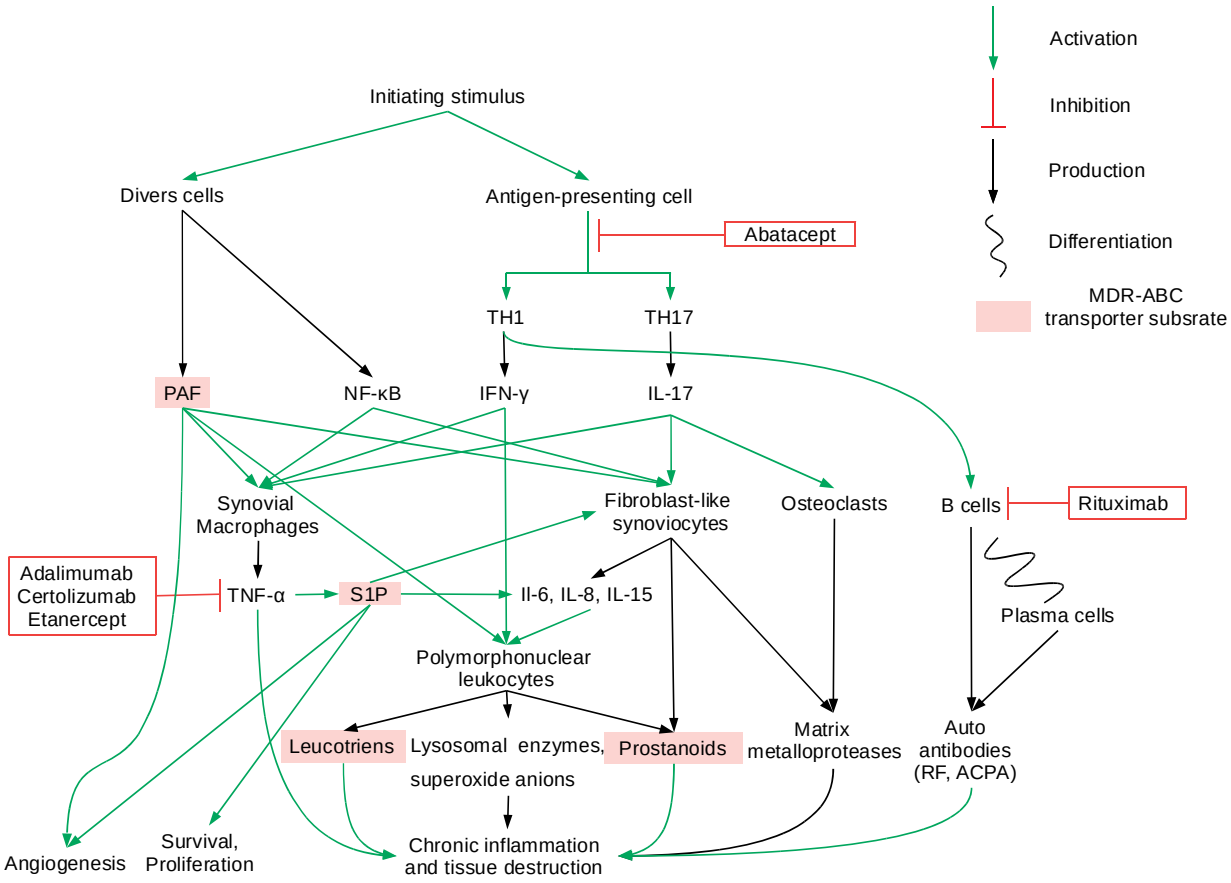

Figure 1. *Simplified schematic view of interaction of MDR—ABC transporters in RA pathomechanism and targets of biological therapeutics.*

The trigger of pathogenesis of RA is still obscure. Th1 and Th17 cells produce various inflammatory cytokines upon interaction with antigen presenting cells. Th1 cells activate B cells to produce auto-antibodies (e.g. rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) [64]. Activated B cells differentiate into plasma cells that produce large quantities of these antibodies. The biological drug Abatacept is a T-cell activation inhibitor. Rituximab, the anti cluster of differentiation-20 (CD-20) antibody efficiently deletes B cells [65]. Th1, Th17 also targets synovial macrophages producers of TNF. The T cell macrophage interaction is mediated via secreted cytokines, interferon (IFN)-gamma and interleukin (IL-17) respectively. IL-17 activates fibroblast-like synoviocytes (FLS) and osteoclasts, two effector cell types in tissue degradation secreting matrix metalloproteases and invading cartilage [67]. FLS exacerbates inflammation due to production of prostaglandins and leukotrienes as well as direct tissue damage via released

lysosomal enzymes and superoxide anions [69]. Nuclear factor-κB (NF-κB) activation in synovial macrophages and FLSs not only enhances the production of pro-inflammatory cytokines and matrix metalloproteinases, but also promotes proliferation and inhibits apoptosis, which leads to disease progression. MDR1 transports platelet-activating factor (PAF) produced by various cell types[25], Transport of PAF may facilitate angiogenesis [28] PAF activates Synovial macrophages, Fibroblast-like synoviocytes and Polmorphonuclear leukocytes. MRP1 transports important mediators of inflammation prostaglandins and leukotrienes. [15]. MRP1 and BCRP transport sphingosine-1-phosphate (S1P) [31] S1P facilitates cell growth, survival, proliferation and angiogenesis. S1P increase cytokine / chemokine production [32]. Anti-tumour necrosis factor (TNF can activate S1P production. TNF-α is inhibited by biological drugs (e.g adalimumab, certolizumab pegol, etanercept). (The author's own illustration)

Llorente et al. found higher MDR1 activity in refractory RA than non-refractory group [72]. Agarwal et al. established the percentage of cells expressing MDR1 were significantly higher in the methotrexate -naive and methotrexate -refractory groups than the healthy controls at baseline, and they also found that the expression of MDR1 in RA correlated with disease activity status[73]. Tsujimara et al. reported that etanercept therapy to inhibit TNFa in refractory RA resulted in an almost complete disappearance of the MDR1- and CD69- high-expressing subgroups [74]. Etanercept, also reduced MDR1 expression and restored intracellular dexamethasone levels of non responder RA patients within two weeks [86] Moreover, Heijden et al. revealed that BCRP may play a role in the efficacy of specific other synthetic DMARDs in RA treatment besides MDR1. Since methotrexate is an in vitro substrate for both BCRP and MRP1, and leflunomide is a high-affinity substrate for BCRP, these transporters may contribute to the reduced therapeutic efficacy of these therapies [75]. BCRP has also been shown to be overexpressed in synovial macrophages particularly in resistant cases [75]. The relevance of BCRP may be partly underscored by the findings that many DMARDs such as methotrexate [45], sulfasalazine [46] and leflunomide [47] are BCRP substrates. Some steroid anti-inflammatory drugs such as glucocorticoids (hydrocortisone, dexamethasone), important therapeutic agents in RA have also been shown to be MDR1 substrates [39, 44]

Wolf et al. found that the determination of MDR may predict responsiveness to methotrexate [75]. Monitoring MDR in RA may have several benefits: it can be used as a disease activity marker in addition to disease activity score 28 joint count (DAS28), CD69 measurements in Phase I, Phase II or Phase III according to European League Against Rheumatism (EULAR) repeated MDR measurements applied monthly can be used to monitor therapeutic efficacy[78]. If MDR1 activity is

decreasing over the time, it may be interpreted as a positive efficacy marker regarding the given therapy. An increase in MDR1 activity might trigger a therapy change earlier on than based on current disease activity monitoring methods, which would contribute to better patient outcomes.

Early diagnosis and immediate, effective therapy are crucial in order to prevent joint deterioration, functional disability and unfavorable disease outcome [77,78]. The optimal management of RA is needed within 3-6 months after the onset of disease, therefore a very narrow „window of opportunity" is present to achieve remission [79] or at least low disease activity (LDA) [80, 81]. It is very important to predict the efficacy of expensive biologics at early stages of treatment [82]. Based on data from a single university center 17.7% of RA patients never achieved low disease activity regardless of receiving all bDMARD [134]. Despite of new generation of drugs there are no validated circulating biomarkers of prognostic use nor are there biomarkers to predict response to specific therapies [84].

1.7. MDR—ABC transporters in RA – expression and pharmacogenetics

The role of transporters in RA has been studied for almost two decades. Most of the studies focused on MDR1 as the prototype ABC transporter and were trying to correlate MDR1 expression with disease status and more importantly drug resistance. These studies with one exception [87] have found increased levels of MDR1 expression in peripheral blood lymphocytes (PBL) or peripheral blood mononuclear cells (PBMC) that correlated with lower intracellular dexamethasone levels in these cells [88, 89]. Moreover, MDR1 activity was higher in refractory than in non-refractory subgroups measured on CD4+ and CD19+ cells [90] and measured on total PBMCs [91].

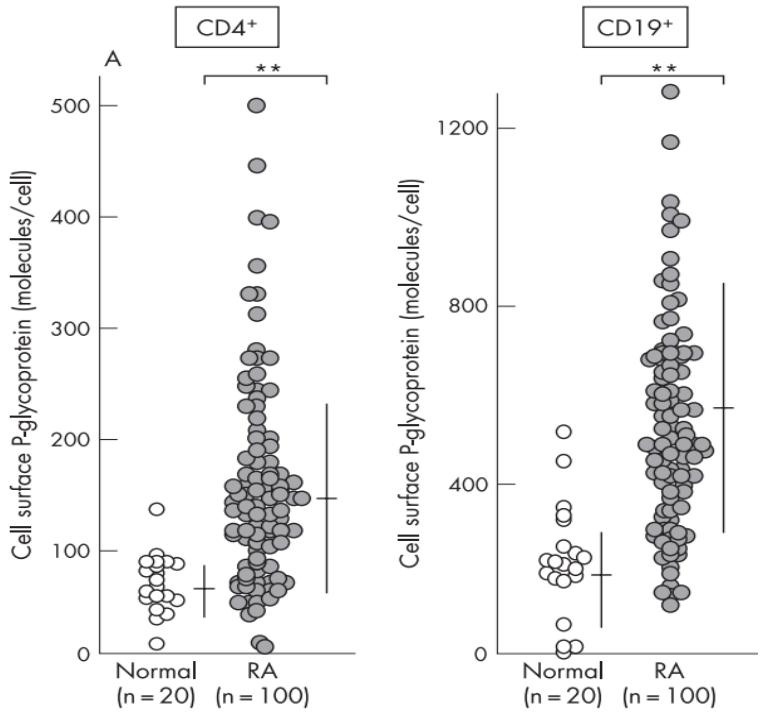

Figure 2. *Expression of MDR1 on lymphocytes from patients with rheumatoid arthritis (RA), as determined by flow cytometry. MDR1 expression on peripheral CD4+ and CD19+ peripheral blood lymphocytes from 20 normal volunteers (open circles) and 100 RA patients (dotted circles).[90 / Figure 1]*

Clinical value of MDR1 expression levels for predicting the risk of DMARD failure (persistent DAS28 3.2.) was measured in serum. Serum MDR1 levels greater than 142 ng/mL had a sensitivity of 78% for detecting nonresponders for DMARD treatment, indicating a 22% possibility for false negatives [135].

In contrast, no correlation was seen between MDR1 expression and disease activity in synovial cells [92], though prior treatments may induce MDR1 expression both in lymphocytes [87] and synovial cells [92] and published data were not always correlated for this important covariate. Dependence of MDR1 activity on the genotype is controversial. Tumour cells of B cell chronic lymphocytic leukaemia patients of 3435CC genotype were shown to have greater MDR1 activity than carriers of the T-allele [93] while no difference was observed in PBMCs from healthy volunteers [94]. No difference in representation of variants between patients and controls was shown [95]. However, probability of remission upon methotrexate and glucocorticoid co-administration was significantly higher in patients of 3435TT genotype than in carriers of the C allele [95, 96]. Conversely, methotrexate monotherapy leads to statistically significantly more nonresponders in the 3435TT

cases than in the 3435CC cases [97]. Methotrexate treatment reduced MDR1 expression levels on lymphocytes [90]. Unexpectedly, methotrexate and / or folate treatment lead to downregulation of MRP1 [98]. No difference was observed in MRP1 status of RA patients and controls [98]. On the contrary, BCRP expression was 2-fold higher in synovial macrophages of RA patients than in controls and a 3-fold increase was observed in nonresponders over responders to methotrexate and/ or leflunomide [74]. Intriguingly, combination therapies of the BCRP substrate methotrexate with other BCRP substrate and/or inhibitor DMARDS (sulfasalazine, leflunomide, hydroxychloroquine, cyclosporine A) yielded better response rates than the monotherapy [48]. But no difference was observed when methotrexate was co-administered with BCRP non-interactors such as azathioprine and gold [48]. In summary, the BCRP data clearly show the importance of this transporter in pathogenesis as well as therapeutic response of the disease. The fact that most small molecular DMARDs are BCRP substrates substantiates the importance of BCRP in RA. The MDR1 data are somewhat controversial. The controversy may stem from the fact that methotrexate, the drug used in most studies is not an MDR1 substrate and/or inhibitor. Early data suggested that methotrexate showed an MDR1 dependent cytotoxicity [99] but substrate nature of methotrexate has not been confirmed in bona fide transport experiments [100]. Nonetheless, MDR1 may play a role through a mechanism other than drug transport as treatment-induced down-regulation of MDR1 correlated with decreased secretion of cytokines in patients [100] and administration of small interfering RNA siMDR1 reduced synovial cytokine production in vitro and in vivo in rat [101].

1.8. Need for biomarkers in RA disease management

Disease management of RA is a costly and challenging exercise for a notable part of patients.

In 2018 in the USA the average direct cost of treatment for RA is 12.509 USD/year for synthetic DMARDs and f 36.503 USD/year for biologic DMARDs [102]. Methotrexate, other synthetic DMARDs and numerous new biological DMARDs are used in monotherapy or in combination. Despite the new generations of drugs, there still remains a large unmet patient need in the treatment of RA[134].

RA is a disease with a highly variable prognosis, quickly leading to disability in many cases.

As it is stated in the EULAR and National Institute for Health and Clinical Excellence (NICE) therapeutic guidelines [78,103] the primary target should be disease remission; however, low disease activity may be an acceptable alternative therapeutic goal. Until the desired treatment target is reached (usually 1–6 months), drug therapy should be adjusted at least every 3 months.

All current therapies have poor response rates and serious side effects, particularly if the first treatment is delayed, and there is no predictive biomarker available for drug efficiency to choose between therapies upfront.

- Methotrexate remains the first choice in early RA treatment showing remission-like response rates up to 50% [104], however, at least 10-30% of patients suffer from various side effects [105].
- Other synthetic DMARDS: half of all treatments had been discontinued by 16 months [106] due to lack of efficacy (25% of all prescriptions and 37% of all discontinuations), followed by toxicity (20% of all prescriptions and 46% of all discontinuations).
- Biological DMARDs: Up to 50% of patients do not respond[107].

There is no good disease activity marker available on the market to show drug response and change the therapy early on.
Regular monitoring of disease activity is particularly important, especially, in patients with high disease activity, there is a need for monthly assessment of the disease status [78]. This effort is currently best supported by composite scoring disease activity evaluation tools (e.g. DAS28, American College of Rheumatology (ACR), simple disease activity index (SDAI), clinical disease activity index (CDAI)). Despite the emerging pool of disease activity biomarkers [108, 109] only a few made it to clinical acceptance so far: autoantibodies like RF, anti-cyclic citrullinated peptide (anti-CCP) and inflammatory markers (e.g. CRP, IL6).

In Phase I of the EULAR guideline, methotrexate is clearly the first choice drug. In case of methotrexate contraindications (or intolerance) the EULAR recommends the following other synthetic DMARDs: leflunomide, sulfasalazine or injectable gold [57]. In this phase, there is a huge need for a good predictive biomarker.

In Phase II, prognostic factors are considered, and monotherapy with methotrexate is not advised any more. Other synthetic DMARDs may be changed or added for patients depending on the choice of sequential monotherapy or step-up combination (involving biological DMARDs) strategy [110]. In case of repeated failures it can take 12 months for a patient to proceed to Phase III from the initial therapy. In this phase, scrutiny of disease activity/progression assessment is essential as 50% of RA patients in this phase tend to encounter disabilities resulting in hospitalizations and sick leaves.

In Phase III, if the addition of TNF-inhibitor is ineffective or causes toxicity, it is advised to change it to other biological DMARDs (e.g. abatacept, rituximab or tocilizumab). In some biological DMARD treatment refractory severe cases of RA, other immunosuppressive agents, such as azathioprine, cyclosporin A, exceptionally, cyclophosphamide may be applied to reduce the disease progression. In this last phase of the disease management, treatment options are limited and very expensive, and in the same time the disease deterioration is irreversible. Given that 50% of the patients do not respond to biological DMARDs and drug response rates highly vary in responders, the measurement of disease activity and simultaneous response/efficiency is of the utmost importance. Literature data suggest that mode of action / efficacy markers would be therapeutically relevant to develop [107].

The best advice on rational RA therapy design is currently incorporated in the therapy guidelines, however they do not contain generally appropriate biomarker utility for therapeutic changes.
It is important to stress that time dimension is very important in RA, since up to a third of patients with a paid job end up work-disabled within 2 years from disease onset, putting a very high burden on medical and social care.

1.9. MDR proteins as biomarkers in RA

Historically, the multidrug resistance (MDR) phenomenon has been proven in hematological malignancies, where the prevalence of efflux transporter (e.g. MDR1, MRP1, BCRP) related drug resistance is about 40% [111]. In the last decades several studies examined the possible role of MDR proteins in autoimmune disorders e.g. RA and focused on the correlation with disease activity, therapy responsiveness and progression (outcome).
MDR expression may correlate with RA disease activity, as well as with responses to methotrexate and other DMARD treatment [73]. There have been very little data available on the possible association between MDR activity and responses to biologics.

1.10. MultidrugQuant™ Kit

MultidrugQuant™ Kit (MDQuest Kft, Szeged Hungary) is the first commercially available, *In vitro* approved clinical diagnostics (IVD) for the detection of MDR protein function (MDR1, MRP1, and BCRP) by quantitative flow cytometry. The transporter activities measured in parallel give a valuable insight about the phenotype of the given cell population. [76, 111]

The assay utilizes fluorescent substrates that readily penetrates into the cell[42]. This proprietary technology has several advantages against other fluorescent dye accumulation tests: it is quick, quantitative, selective for the above mentioned ABC transporters [112] , furthermore, it is normalized to an internal standard minimizing batch – to – batch and interlab variations.

In case of active BCRP measurements, intracellular accumulation of fluorescent signal occurs after applying BCRP-specific substrate (mitoxantrone) in the presence and the absence of BCRP inhibitor (KO134). For the functional measurements of MDR1 and MRP1 activities, MultidrugQuant™ Kit also utilizes a non-fluorescent precursor substrate (calcein acetoxy methyl ester (calcein-AM). The cleavage of this substrate by endogenous esterases results in a non membrane permeable fluorescent derivate trapped in the cytoplasm due to its hydrophilicity [42]. Beside the previously mentioned advantage of this assay it is the relatively insensitive to changes to other cellular parameters, including intracellular pH, Ca^{2+} and Mg^{2+} concentrations [112]. As calcein-AM is an excellent substrate for both MDR1 and MRP1, they remove the non-fluorescent precursor before the non-specific esterases could cleave the acetoxy methyl ester group producing the free, fluorescent calcein [40]. Thus, the activity of these transporters results in lower intracellular accumulation of the fluorescent derivate. Consequently, the more active MDR proteins are, the less fluorescent derivate accumulates in the cell. In MDR-expressing cells, the addition of verapamil an inhibitor both for MDR1 and MRP1 and indomethacin a selective inhibitors of MRP1 block the dye extrusion activity of the relevant transporter, and increases dye accumulation in the cells. In the absence of significant MDR—ABC transporter activity, a lack of transporter-mediated efflux means that the net dye accumulation is faster in the cells, which in turn is not influenced by the presence of an MDR—ABC transporter inhibitor or substrate.

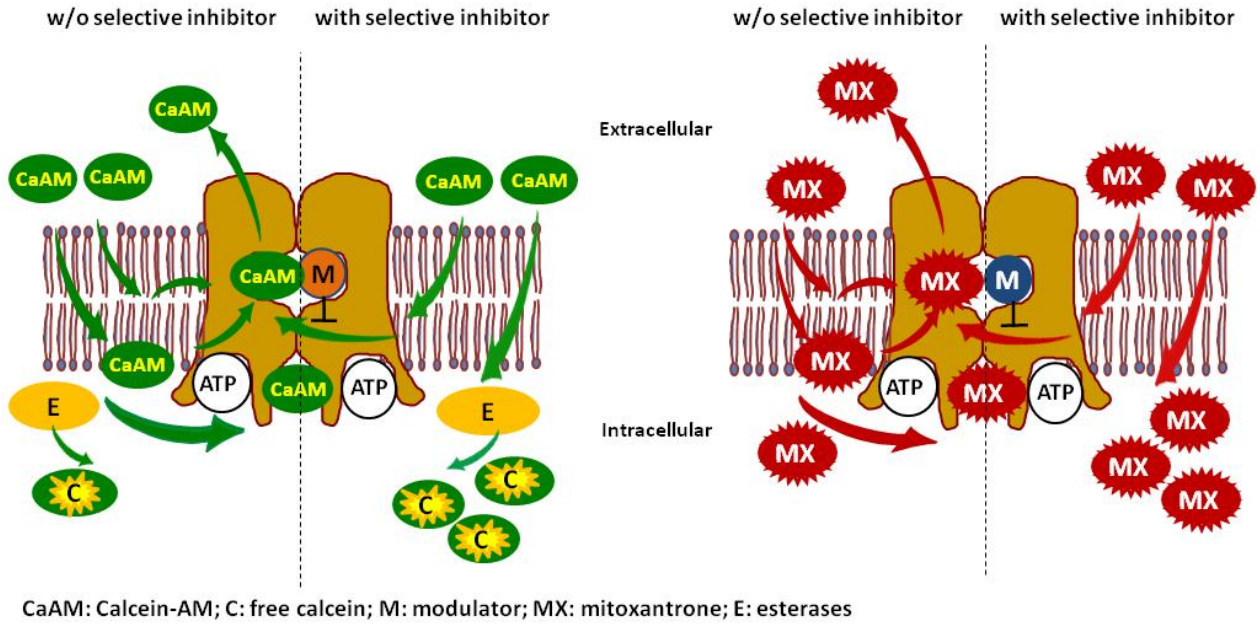

Figure 3. *Dye efflux assays applied in the MultidrugQuant™ Kit. The calcein-assay (left panel) is based on determining fluorescence intensity using a flow cytometer. After short in vitro incubation of the cell suspension with the fluorogenic dye calcein-acetoxymethyl ester (calcein AM), activity MDR1 and MRP1 is determined using selective inhibitors of the transporters. Intracellularly calcein-AM is rapidly hydrolysed by esterases to yield the highly fluorescent free acid, calcein, which due to its hydrophilic character becomes trapped in the cytoplasm. The Mitoxantrone-assay (right panel) measures the activity of BCRP transporter applying a similar principle. The assay utilises mitoxantrone as a fluorescent dye and Ko134 as an BCRP-specific inhibitor. Please note that mitoxantrone is fluorescent and, therefore, does not require further intracellular processing. [38 / Figure 1.]*

Respective activities of multidrug transporters are reflected by the difference between the amount of fluorescent dye accumulated in the presence or absence of the selective inhibitors. This difference is normalized to the dye uptake measured in the presence of the inhibitor, thus, the result of the test is independent from any non-MDR—ABC transporter-mediated factors, like the differences in the cellular properties (membrane lipid composition, intracellular esterase activity, cell size, cell surface, etc.); and the methodological differences (e.g. use of different equipment, amplification, and individual variables). The results of the assay are expressed in MDR Activity Factor (MAF) values, determined from correlated clinical data. Since the influence of these non-MDR—ABC transporter-mediated factors is reduced by the normalization approach intra- and interlaboratory comparison of MAF values is feasible. Selective inhibitors can be used to distinguish between the transport activity of MDR1 and MRP1. The kit component for the inhibition of both MDR1- and

MRP1-mediated dye effluxes providing a dye accumulation rate that can be used for standardization, while another component provided by the MultidrugQuant™ Kit selectively blocks the activity of MRP1. After a short, simple calculation, separate measurements of multidrug resistance for both MDR1 and MRP1 activities can be obtained. [113]

The exact calculation of MAF values are presented in the Materials and Methods / Flow Cytometry section.

For the appropriate investigation of primary cells, proper cell specific fluorescent immunocytochemistry labelling should be performed. In this case, cell labelling must be carried out after performing MultidrugQuant™ Kit assay measurements in neither fixed, nor permeabilized cell populations.

2. Aims

The importance of MDR—ABC transporters in RA is well published. However, that MDR—ABC transporter activity may be of predictive value for biological therapies has not been established.

The aim of this project was to gain an understanding of the characteristics of MDR—ABC transporter activity in normal population and in RA patients. And establish it as a predictive biomarker in biological therapy.

We investigated

1. The activity of three clinically relevant transporter, MDR1, MRP1 and BCRP in CD3+ lymphocytes from healthy volunteers in order to describe normal reference values.
2. The effects of gender and age on transporter activity reference values.
3. Is there a change in MDR1, MRP1 and BCRP activity in CD3+ and CD19+ lymphocytes from RA patients during biological therapy?
4. How to utilize the different transporter activity characteristics as a predictive tool of biological therapeutic response in patients before as well as 4 to 6 and 12 weeks after the initiation of biological therapy.

3. Materials and Methods

3.1. Patient Recruitment

The reference MDR activity factor (MAF) intervals were determined according to the Clinical Laboratory Standards Institute (former NCCLS) (CLSI) guideline C28-A2 [114] on CD3+ lymphocytes of a reference population of 120 healthy volunteers. (IVDMDQ08 Study between 2008-2013. contract research organization (CRO): M.E. Trial Masters Kft. Principle Investigator: J. Kappelmayer MD, PhD, Dsc University of Debrecen) The study protocol and the written informed consent form had ethical approval from the Medical Research Council Ethics Committee for Clinical Research (ETT TUKEB) of Hungary. The study adhered to the tenets of the most recent revision of the Declaration of Helsinki. Based on normal values of full blood count, the CD4/CD8 ratio, liver and kidney function tests, 120 healthy subjects aged 18 to 74 years were selected. The age distribution was 18—39 years for 49 subjects, 40—59 years for 45 subjects and 60—74 years for 26 subjects. The gender distribution was 58 female and 62 male subjects. There was no observed adverse event or side effect in this study.

The MDR activity factor (MAF) intervals were determined on CD3+ and CD19+ lymphocytes for 39 RA patients recruited at the outpatient clinics of the Department of Rheumatology, University of Debrecen, Hungary and the Department of Rheumatology and Clinical Immunology, Charité, Berlin, Germany. Patients were sampled before the start of biological treatment as well as between 4 and 6 weeks and at 12 weeks of treatment. DAS28 and C-reactive protein (CRP) values were also recorded at this time. Patients were regarded as nonresponders (n = 12) if DAS28 values showed a decrease of less than 25% between the start of biologicals and at 12 weeks of treatment (arbitrary cut-off). Patient characteristics as well as details of the therapy received are included in Table 2 .

	Healthy controls (n = 35)	Responder (n = 27)	Nonresponder (n = 12)
Age (years)	54 (42-62)	56 (49-61)	51 (39-61)
Gender (male/female)	4/31	2/25	1/11
RA duration (years)	-	10 (5-14)	8.5 (5-15)
No. of patients receiving			
methotrexate	-	15 (56%)	6 (50%)
prednisolone	-	9 (33%)	5 (42%)
adalimumab (TNF inhibitor)	-	2 (7%)	1 (8%)
certolizumab pegol (TNF inhibitor)	-	5 (19%)	3 (25%)
etanercept (TNF inhibitor)	-	7 (26%)	3 (25%)
abatacept (T-cell inhibitor)	-	13 (48%)	5 (42%)

Table 2. *Clinical characteristics of Responder and Nonresponder RA patients as well as healthy controls. Data are expressed as median (Interquartile Range (IQR)) for continuous variables and as number (percentage) for categorical variables. n- sample size [2 / Table 1.]*

Healthy controls (n = 35) were sampled at the Department of Rheumatology, University of Debrecen, Hungary on a single occasion. They had a negative history of autoimmune disorders including RA and a negative status upon physical examination as well as no infectious symptoms within three weeks before sampling.

Exclusion criteria for all participants included chronic infectious diseases requiring systemic treatment, autoimmune diseases other than RA, immunodeficiencies, allergic diseases and hematological malignancies or solid tumors.

Written informed consent was obtained from all participants and the study adhered to the tenets of the most recent revision of the Declaration of Helsinki. Ethical approval for the study was granted by local ethics committees of the University of Debrecen (TUKEB 21018/2014/EKU) and Charité (EA1/193/10), respectively.

3.2 Peripheral Blood Mononuclear Cell (PBMC) isolation

From volunteers for the reference value 3 ml uncoagulated and 6 ml Ethylenediaminetetraacetic acid sodium (K3EDTA) anticoagulated, from the RA patients 6 ml K3EDTA anticoagulated peripheral blood samples were collected at the time of examination. Heparin is known to interfere with the activity of MDR proteins, therefore the use of heparinized blood is not recommended (Ref 28). PBMCs were separated by density gradient centrifugation using Ficoll Histopaque-1077 (Cat. No: H8889, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions.

Since this is an ATP-dependent functional assay, it requires cells in good condition not depleted of intracellular energy stores. Therefore, blood samples were processed within six hours following sampling and stored at room temperature before processing.

3.3 Flow Cytometry

Measurements were conducted on a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) equipped with 488 nm and 635 nm lasers or on a Miltenyi MACSQuant flow cytometer, equipped with 405nm, 488 nm and 638 nm lasers, respectively.

The MultidrugQuant™ Kit (Catalogue Number: MDQ101D) was used strictly following the manufacturer's instructions. Separated PBMCs were washed twice with 5 ml of Hanks' Balanced Salt Solution (HBSS) by centrifugation at 300x gravitational force equivalent (g) for 10 minutes. Supernatants were discarded and cells were counted. A cell suspension containing 2-5x10^6 cells was prepared using HBSS and 800 ul of cell suspension was added into 15 tubes.

In this assay, fluorescent reporter substrates are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of highly selective inhibitors, allowing for quantitative, standardized assessment. PBMCs were loaded with fluorescent MDR activity reporter substrates (1.25 uM working solution of calcein-AM for MDR1 and MRP1, em: 515 nm, incubated for exactly 10 minutes followed by rapid centrifugation and 1 mM working solution of mitoxantrone for BCRP, em: 684 nm, respectively, incubated for exactly 30 minutes followed by rapid centrifugation) and treated with highly selective MDR protein inhibitors (12.7 mM working solution of verapamil for MDR1 and MRP1, 2 mM working solution of indomethacin for MRP1 and 0.1 mM working solution of KO134 for BCRP, respectively) to obtain multidrug activity factor (MAF) values. Note that the above working concentrations are further diluted upon addition to the cell suspension to avoid cell toxicity as described in the manufacturer's instructions.

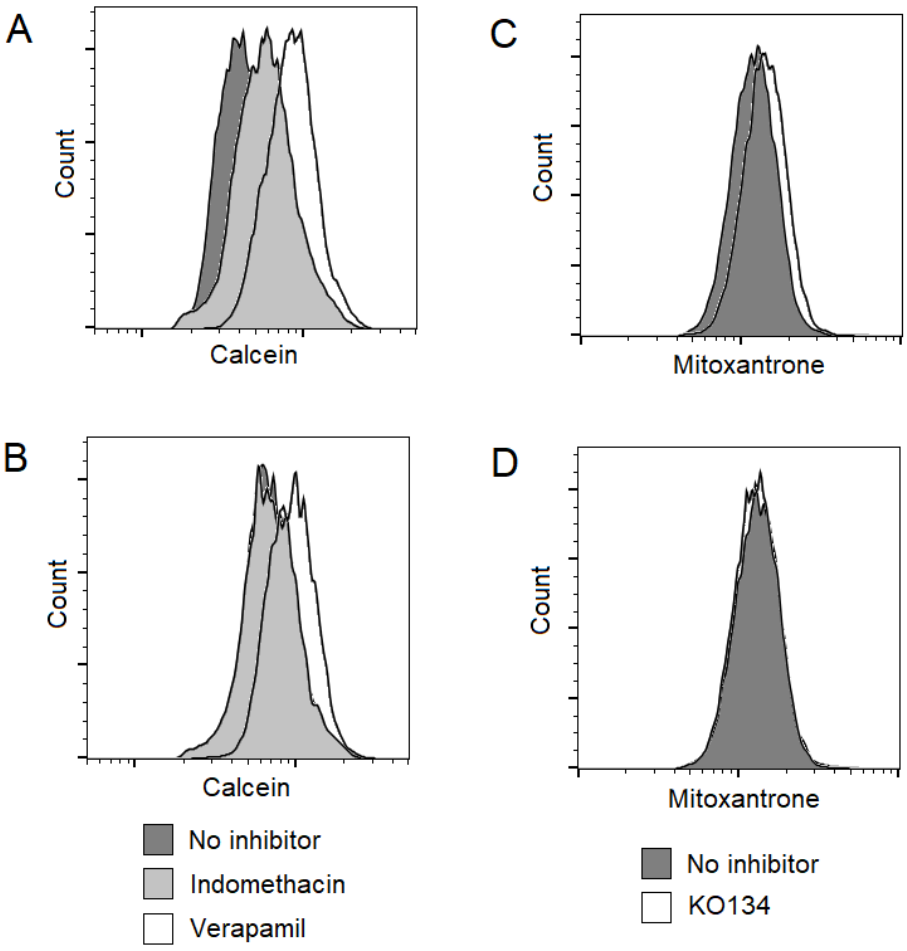

Figure 4. *The effects of highly selective inhibitors of MRP1 (indomethacin) and MRP1 + MDR1 (verapamil) on calcein substrate fluorescence intensity at high (A) and low (B) transporter activity, and the effects of the highly selective inhibitor of BCRP (KO134) on mitoxantrone substrate fluorescence intensity at high (C) and low (D) transporter activity in CD3+ cells – representative samples of RA patients [2 / Figure 1.]*

Cell surface staining was applied to select CD3+ cells from volunteers for the reference value and CD3+ and CD19+ cells from RA patients using anti-human CD3+-PerCP and CD19+-PE monoclonal antibodies (Cat. No: 345766 and 345789, respectively, both BD Biosciences) in case of calcein-AM stained cells and anti-human CD3+-FITC (fluorescein isothiocyanate) and CD19+-PE (phycoerythrin) monoclonal antibodies (Cat. No: 345764 and 345789, respectively, both BD Biosciences) in case of mitoxantrone stained cells according to the manufacturer's instructions. Cells were centrifuged at 2000x g for 1 min. Supernatant was discarded and cells were resuspended in 500 ul of HBSS and run on the flow cytometer immediately.

Activities of multidrug transporters are reflected by the difference between the amount of calcein/mitoxantrone accumulated in the presence or absence of the selective inhibitor(s). When calculating the MAF values, this accumulation difference is normalized to the dye uptake measured in the presence of the inhibitor. Thus, the result of the test becomes independent from factors influencing the cellular accumulation of calcein other than the activity of the multidrug transporters.MAF values were calculated using medians of geometric mean fluorescent intensities (MFIs) of the replicates from the difference between MFIs of cells with and without the specific inhibitors, respectively (See the calculation in the MultidrugQuant™ Kit section). Measurements were performed in three technical replicates. coefficient variations (CV) of technical replicates were between 0.6-4.1% for volunteers for the reference value and <10% for RA patients. The gating strategy applied is demonstrated on a representative sample in Figure 5.

MAFC = 100 x (Fmax – Fo) / Fmax
MAF of MRP1 = 100 x (FMRP1 – Fo) / Fmax
MAF of MDR1 = MAFC – MAF of MRP1
MAF of BCRP = 100 x (FMX – F0) / FMX
Fmax/FMX: Calcein/mitoxantrone fluorescence with verapamil or KO134, respectively
Fo: fluorescence without inhibitor
FMRP1: Calcein fluorescence with indomethacin

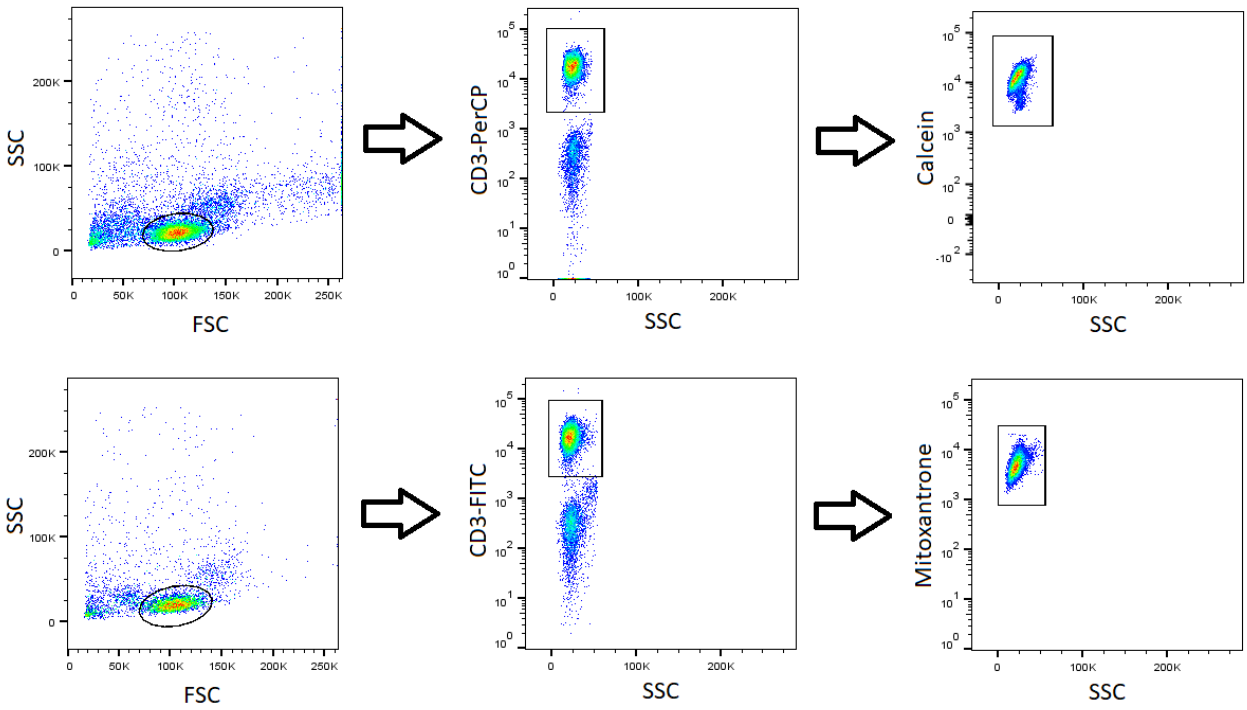

Figure 5: *Gating strategy to determine calcein and mitoxantrone fluorescence on a representative sample. Forward scatter characteristics (FSC), side scatter characteristics (SSC). [1 / Figure 1.]*

3.4. Statistics

Among volunteers for the reference value determination the distribution of MAFC and MAFMDR1 values are acceptable as normal according to the Shapiro-Wilk test ($p > 0.05$) with no outlier data (Grubbs test, $p > 0.05$). For MAFMRP1 values the distribution appears non-normal (Shapiro-Wilk test, $p < 0.05$) but there are no outlier values (Grubbs test, $p > 0.05$). MAFBCRP values also show non-normal distribution with three outlier values. The deviation from the normal distribution can be explained partly by the multiple “0” values obtained for MAF.

We applied Univariate Tests of Significance to correlate MAF values with the age of the studied subjects using Sigma-restricted parameterization. Because the gender distribution of MAF values was normal, Student's t-tests and F-tests were used for comparisons between male and female subjects.

To see the predictive value of the transporter activity comparisons were made using the Kruskall-Wallis test or the Mann-Whitney test as the distribution of data appeared to be non-normal according to the Shapiro-Wilk test. p values < 0.05 were considered significant. Statistics were calculated using the GraphPad Prism 5 software (La Jolla, CA, USA).

To find an activity threshold value to distinguish between responders and nonresponders the receiver operator curve (ROC) was used. When the results of a diagnostic test are considered to discriminate between two populations (eg. responders versus nonresponders), a perfect separation between the two groups is rarely observed. For every possible cut-off point selected to discriminate between the two populations, there will be some cases with the responder status correctly classified as responder (True Positive), but some responders will be classified into the nonresponder group (False Negative). On the other hand, the majority of nonresponders will be correctly classified as nonresponders (True Negative), but some will be classified as responders (False Positive). In a ROC curve the true positive rate (Sensitivity: calculated as the True Positive / (True Positive + False Negative)) is plotted against the false positive rate (100-Specificity; wherein specificity is calculated as the False Positive / (False Positive + True Negative), i.e. 100-Specificity is the True Negative / (False Positive + True Negative)), demonstrating different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups.

4. Results

In the predictive biomarker assessment study [2] besides the transporter activity measurements as an internal control the biologics therapy efficacy was measured. DAS28 and CRP, values were followed and we compare the clinical and immunological parameters of responder and non responder patients.

Our study confirmed what is written in the literature, that with higher disease activity patients respond better to biologics [79, 80]. Among RA patients DAS28 values decreased upon treatment in responders in contrast to nonresponders. Of note, initial DAS28 values were higher in the responder group compared to nonresponders (Figure 6, Table 3,). Neither differences were observed in CRP values between the two group, nor changes in CRP were demonstrated upon treatment.

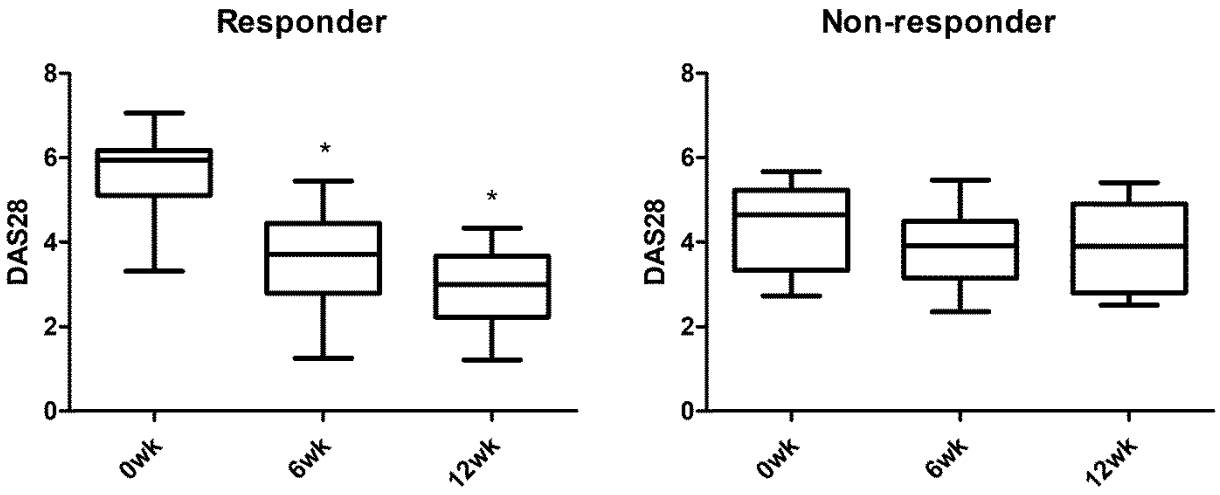

Figure 6. *DAS28 values at different sampling time points in responder and nonresponder RA patients. *p < 0.05 vs. 0wk [2 / Figure 2.]*

Activities of multidrug transporters are reflected by the difference between the amount of calcein/mitoxantrone accumulated in the presence or absence of the selective inhibitor(s). When calculating the MAF values, this accumulation difference is normalized to the dye uptake measured in the presence of the inhibitor. Thus, the result of the test becomes independent from factors influencing the cellular accumulation of calcein other than the activity of the multidrug transporters. MAFC is a composite activity value for the MDR1 and MRP1 transporters.

Control values (Table 3) measured in the predictive biomarker assessment study [2] were within the normal range, established in our reference range study [1].

		0 wk		6 wk		12 wk	
	Control	**Responder**	**Non-responder**	**Responder**	**Non-responder**	**Responder**	**Non-responder**
DAS28	-	5.94 (5.11-6.17)	4.65[b] (3.33-5.23)	3.71[c] (2.79-4.45)	3.93 (3.14-4.50)	3.00 [c] (2.23-3.67)	3.90[b] (2.81-4.90)
CRP	-	11.1 (2.6-16.6)	8.4 (1.4-15.1)	4.4 (1.3-7.9)	4.4 (1.5-10.4)	3.7 (2.1-5.6)	7.5 (2.7-11.6)
CD3+ MAFC	18.3 (14.7-22.9)	18.9 (14.0-25.2)	23.5[b] (17.1-33.7)	17.1 (12.3-22.6)	22.7[b] (16.7-29.2)	18.3 (15.7-24.2)	25.2 (15.9-30.7)
CD3+ MAF MRP	3.1 (1.2-5.7)	4.8 (0.0-8.0)	5.7 (2.2-8.0)	2.2 (0.0-7.9)	8.4[b] (2.1-11.3)	5.7 (3.7-8.5)	7.7[a] (4.0-11.6)
CD3+ MAF MDR	14.6 (12.5-18.1)	12.9 (11.0-16.7)	19.1[b] (11.2-24.0)	12.4 (11.2-15.4)	15.8[b] (14.3-18.7)	12.5 (9.2-17.5)	13.6 (6.0-20.0)
CD3+ MAF BCRP	2.5 (0.8-5.7)	3.1 (0.0-4.4)	5.0 (2.0-8.0)	2.0 (0.0-5.5)	3.9 (2.5-10.7)	1.4 (0.0-4.3)	4.5 (1.8-5.8)
CD19+ MAFC	12.8 (8.9-17.9)	15.1 (8.1-22.1)	20.6 (13.5-31.0)	13.2 (9.3-20.4)	17.6 (11.4-27.2)	17.4 (13.1-22.3)	17.6 (9.2-25.9)
CD19+ MAF MRP	2.2 (0.0-6.3)	0.9 (0.0-7.7)	4.4 (0.0-5.8)	0.6 (0.0-5.1)	6.8[b] (0.5-9.6)	3.2 (0.3-6.8)	5.1 (1.9-10.9)
CD19+ MAF MDR	9.9 (8.0-14.0)	11.1 (6.0-16.3)	15.7 (8.4-25.4)	11.4 (5.3-14.8)	13.6 (8.6-17.7)	14.0 (7.1-17.7)	8.8 (1.9-15.7)
CD19+ MAF BCRP	3.8 (1.0-6.3)	3.1 (0.7-7.0)	4.5 (0.0-11.0)	2.7 (0.0-5.2)	5.0 (3.1-8.4)	2.9 (1.3-5.1)	3.0 (1.8-3.7)

Table 3. *Activity of various MDR transporters on CD3+ and CD19+ cells in RA patients and healthy controls. Data are expressed as median (IQR), $p < 0.05$ [a] vs Control, [b] vs Responder, [c] vs 0 wk value. MAFC – composite multidrug activity factor (of MRP1 and MDR1 activity), MAFMRP– multidrug activity factor of MRP1, MAFMDR – multidrug activity factor of MDR1, MAFBCRP – multidrug activity factor of BCRP*

The reference range study [1] indicates that in a normal population MAF value of MRP1 is 2.5 [0.0 – 12.5] (median [2.5 – 97.5 percentiles]) and are independent from age (Figure 7c). The distribution of the MAF value of BCRP is 3.4 [0.0 – 22.0] (median [2.5 – 97.5 percentiles]) and is also independent from age (Figure 7d). On the other hand, MAFC and MAFMDR1 show negative correlation with the age of the studied subjects ($p = 0.003$, $r = -0.27$ and $p = 0.0001$, $r = -0.34$, respectively) (Figure 7a and 7b). The distribution of MAF value of MDR1 is 12.9 [0.0 – 25.7] (median [2.5 – 97.5 percentiles]) and that of MAFC is 16.5 [0.0 – 32.0] (median [2.5 – 97.5

percentiles]). MDR1 activity greatly contributes to the MAFC value and is therefore likely to be accountable for its similar correlation with age.

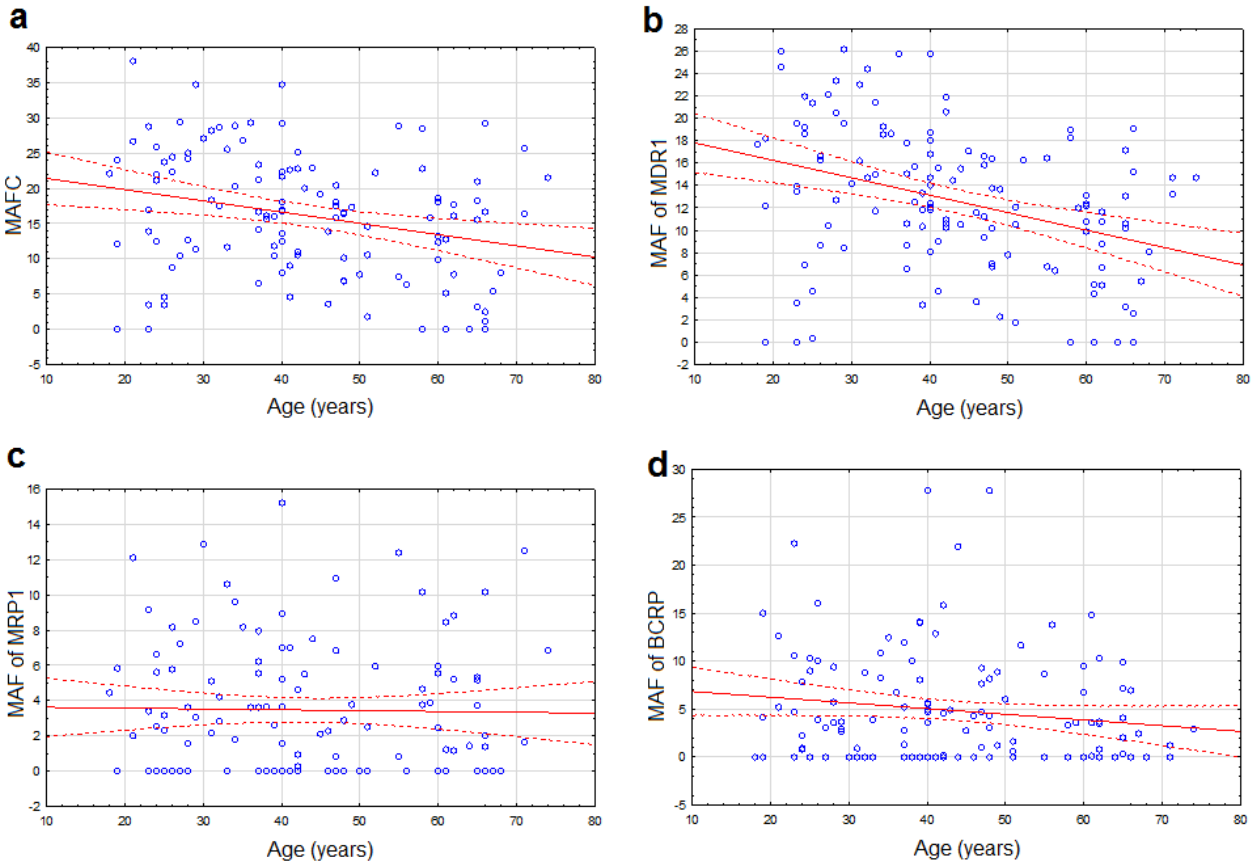

Figure 7. *Scatterplots of the MAFC value (a) and MAF values of MDR1 (b), MRP1 (c) and BCRP (d) against age. 95% confidence interval is represented in red. MAFC and MAFMDR1 show negative correlation with the age of the studied subjects.[1 / Figure 2.]*

MAF values of 3 lymphocytes from RA patients showed the following values: at the time of bDMARD therapy initiation, MAFC values of responders were almost the same as compared with healthy individuals (18.9 vs 18.3), however, in case of nonresponders, MAFC values on 3 lymphocytes were significantly upregulated as compared with controls (23.5 vs 18.3). During bDMARD treatment in case of responders, a slight down regulation was detected 6 weeks after starting therapy, however, in later time points, MAFC value did not showed any alterations as compared with control samples and values at the time of therapy initiation. Importantly, in case of responders, average MAFC values were below the cut-off values at the time of diagnosis and 6 weeks after starting bDMARD treatment. In contrary with responders, MAFC values of

nonresponders were significantly higher as compared with healthy controls at the time of diagnosis (0 weeks, 23.5 vs 18.3). As same as responders, bDMARD treatment had no impact on MAFC values, however, MAFC of CD3+ lymphocytes during bDMARD treatment were significantly higher as compared with healthy volunteers.

Although in case of MAFMDR cut-off value statistical significance was not detected at the time of diagnosis (17.4; 0 weeks), its prognostic value is still high, in particular together with the 6 weeks cut-off value data (13.9). At the time of diagnosis, responder values did not show any alterations as compared with controls, however, MAFMDR values of nonresponders were significantly above the control data (19.1 vs 14.6). Prolonged bDMARD treatment had no significant impact on MAFMDR1values of responders. In case of nonresponders, mild down regulation was detected after starting bDMARD treatment as compared with values at the time of diagnosis.

MAFMRP values has strong prognostic value 6 weeks after starting bDMARD treatment. At the time of diagnosis (0 weeks), mild upregulation was detected in RA patients as compared with healthy controls. 6 weeks after starting bDMARD treatment, a mild down regulation was detected as compared with 0 weeks value. Importantly, opposed to responders, a significant upregulation was detected in nonresponders (2.2 vs 8.4).
When comparing RA patients to control the MAFMRP1 in CD3+ cells was higher at 12wk in nonresponders compared to Controls. (Table 3.)

At 6 weeks MAFMRP values of CD19+ cells were higher in nonresponders compared to responders (Table 3).

No significant changes were demonstrated in MAF values in the respective RA patient groups with the progress of treatment. There is a tendency or signal but it needs further investigation. (Figure 8).

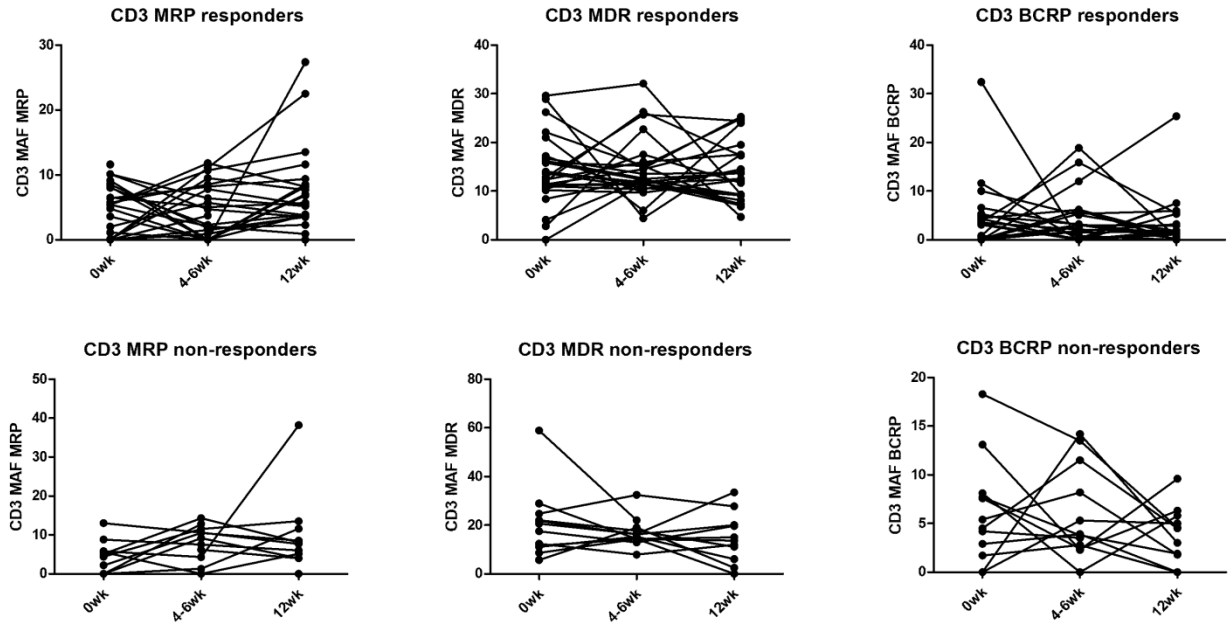

Figure 8. I*ndividual changes of MAF values on CD3+ cells over time in Responder (n = 27) and Nonresponder (n=12) RA patients. No significant changes were demonstrated in MAF values in the respective RA patient groups with the progress of treatment. [2 / Figure 3.]*

No difference was demonstrated in MAFBCRP values in CD3+ or CD19+ cells between control, responders and nonresponders.

No difference was detected in any of the four MAF values between men and women (Table 4). Gender does not affect the presence or lack of correlation between MAF values and age (Figure 9).

	Men (n = 62)			Women (n = 58)		
	Median	2.5 %	97.5%	Median	2.5 %	97.5%
MAFC	16.4	1.2	29.4	16.6	0.0	34.7
MAFMDR1	13.2	0.0	25.7	12.2	0.0	26.0
MAFMRP1	3.0	0.0	10.2	2.1	0.0	12.9
MAFBCRP	4.2	0.0	27.8	2.7	0.0	14.8

Table 4. *Gender-specific MAF values of the investigated MDR—ABC transporters on CD3+ lymphocytes. Data are presented as median [2.5 – 97.5 percentiles]. No statistically significant difference was observed in any of the investigated MAF values between men and women.[1 / Table 1.]*

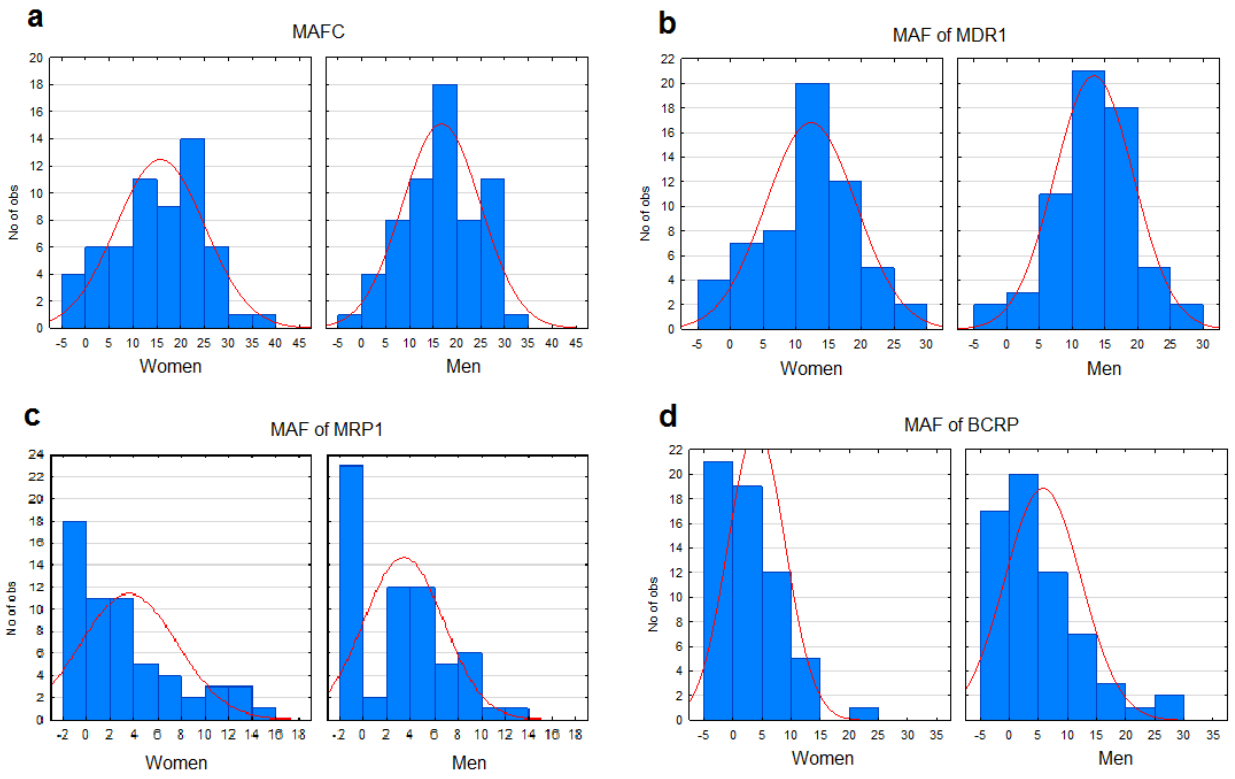

Figure 9. *Histograms representing the distribution of the MAFC value (a) and MAF values of MDR1 (b), MRP1 (c) and BCRP (d) in men and women. Gender does not affect the presence or lack of correlation between MAF values and age.*

Receiver operating characteristic (ROC) analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6wk. Cut-off thresholds were calculated for MAF values with ROCs of adequate probability (p) and area under the curve (AUC) values (Figure 10). Patients with MAF values above the respective cut-off thresholds are likely to be nonresponders to treatment.

Cut-off= 21.3 MAFC of CD3+ cells at 0wk: p = 0.043, AUC = 0.68;

Cut-off= 20.3 MAFC of CD3+ cells at 6wk: p = 0.033, AUC = 0.72;

Cut-off= 6.0 MAFMRP on CD3+ cells at 6wk: p = 0.049, AUC = 0.69,

Cut-off= 13.9 MAFMDR on CD3+ cells at 6wk: p = 0.048, AUC = 0.70.

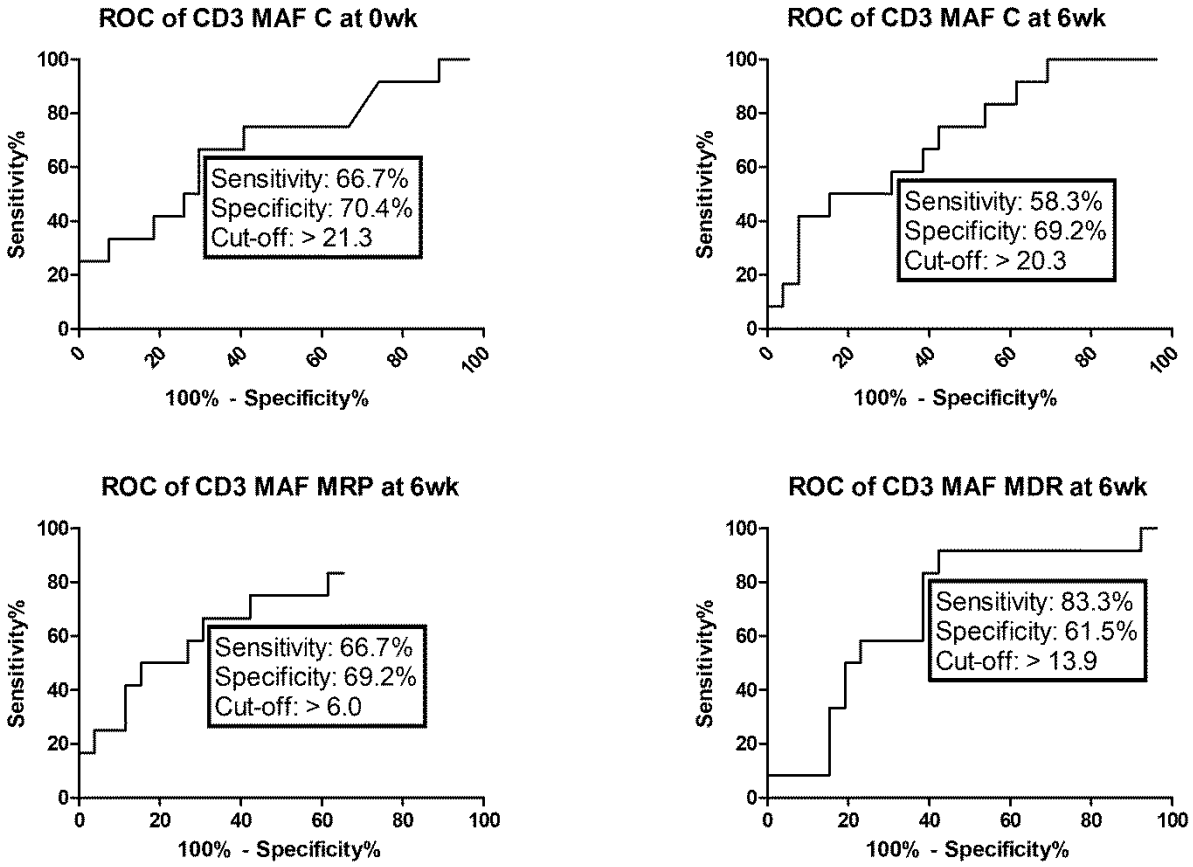

Figure 10. *ROC analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6wk. Patients with MAF values above the respective cut-off thresholds are likely to be nonresponders to treatment. Cut-off= 21.3 MAFC of CD3+ cells at 0wk: p = 0.043, AUC = 0.68; Cut-off= 20.3 MAFC of CD3+ cells at 6wk: p = 0.033, AUC = 0.72;Cut-off= 6.0 MAFMRP on CD3+ cells at 6wk: p = 0.049, AUC = 0.69, Cut-off= 13.9 MAFMDR on CD3+ cells at 6wk: p = 0.048, AUC = 0.70.*

5. Discussion

The work presented in the thesis aimed to explore utilization of flow cytometry to support MDR—ABC-transporter activity-based decision-making in clinical settings.

MDR—ABC transporters play a widespread role in drug resistance that includes drug efflux as well as efflux of physiological substrates affecting immune cell activity. MDR—ABC transporetrs have been considered potential biomarkers in various diseases, RA among them [85]. MDR expression or functionality may correlate with RA disease activity, as well as with responses to methotrexate and other DMARDs [43, 76, 86] and steroid anti-inflammatory drug treatment [39]. Availability of data on possible association between MDR activity and responses to biologics is limited.

Early on evaluation of transporter status was commonly done using qRT-PCR [115-117] With recent development of the massspectrometry-based proteomics methods absolute quantification of membrane transporters has become a common tool [118]. It has also shown that mRNA expression data may not always correlate with protein expression [119].

Even the proteomics methods carry a chance for variation as there are differences in experimental methods, work-up of tissue / cell specimen, utilization of markers to make sure membranes from the relevant compartment/domain (i.e. plasma membrane) are measured [118]. In addition, mass spectrometry-based proteomics requires equipment and experience not commonly available in clinical labs.

Therefore, activity data such as data presented here compares favorably to other options to determine transporter-mediated MDR. Evaluation of MDR activity in a clinical setting assumes determination of the normal range for healthy controls. This was also done and data are presented in the thesis. [1].

The work presented utilized the MultidrugQuant™ Kit. In the MultidrugQuant™ Kit assay, fluorescent reporter substrates are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of specific inhibitors. This novel method offers a standardized approach to measure MAF of the three clinically most relevant MDR—ABC transporters (MDR1, MRP1 and BCRP). Although it is primarily suitable for the determination of MAF values of peripheral blood or bone marrow cells on the flow cytometry platform, any cell types that are stable in a suspension could be used for measurements. However, taking into account the easy accessibility of peripheral blood from patients, the clinically most relevant utilization of this method may be oncohematology and immunology. The calculation of MAF values is

independent from variability caused by inter-assay, equipment or lab environment factors, as detailed in the Methods section. A further great benefit of the method is the flexibility regarding the number of cell types analyzed depending on the fluorescent cell surface markers used, offering a comprehensive approach for the assessment of transporter activity in multiple clinically relevant cell subsets at the same time.

MAF of MDR1, and consequently the MAFC value, which consists of the functional activity of both MDR1 and MRP1, decrease with age. While this has not been demonstrated earlier in T lymphocytes, decreased MDR1 function with aging in the blood–brain barrier (BBB) has been described by both van Assema et al. and Bauer et al. verified by positron emission tomography (PET) studies [120,121]and by microdialysis in rats [122]. On the contrary, no effect of aging was shown on MDR1 protein expression in hepatocytes [43]. These findings demonstrate the importance of tissue specificity regarding MDR1 functionality, potentially affecting the design of clinical trials, as well as diagnosis and treatment of patients throughout different age groups. Drugs that are substrates of the MDR1 protein may therefore need to be administered in different doses depending on age to achieve the same therapeutic effect.

A potential extension of our current study may be the determination of MAF values of the investigated transporters, as well as their age and gender dependence in other cell and tissue types, especially those responsible for drug metabolism, such as hepatocytes and renal cells.

In contrast with age, no effect of gender was noted on the activity of the studied transporters, as MAF values of MDR1, MRP1 and BCRP, as well as the MAFC value are consistent across both male and female subjects. For MDR1 this is consistent with earlier findings utilizing mass-spectrometry-based proteomics [43].

Thus, our findings demonstrate that the determination of the functional activity of MDR—ABC transporters is achievable using a flow cytometry - based standardized method and complements other investigation modalities, such as expression levels of the transporters. Having established the normal range of MAF values on CD3+ lymphocytes of a healthy population, our results enable investigators to study the functional activity of MAF in different disease states, such as leukemia or autoimmune disorders, allowing for the development of novel flow cytometry based diagnostic tools. In the future, these tools may help to improve disease activity and therapeutic response monitoring in the clinical setting.

Our work is an important development demonstrating that flow cytometry is a growing field with important clinical applications to cellular [123, 124] as well as extracellular vesicle measurements [125].

Role of transporters in conventional synthetic and biological therapy of rheumatoid arthritis is the subject of extensive current research. In recent publications Muto et.al 2021, described that the good response to MTX is associated with a decrease in the expression of ABCG2, the BCRP coding gene, in patients with RA. [126]. Gao et.al 2020, reviwed the expression of drug transporters in drug-resistant and drug-sensitive patients, and abnormal transporter expression and transport activity have been found in patients with MTX resistance [127]. Zhang et.al 2020, reviewed the efficacy of bDMARDs and concluded that MDR—ABC transporter activity is a potential biomarker for response to DMARD adding that response is also affected by personal factors, for example, age, smoking, body mass index, immunogenicity, and genetic polymorphisms. [128]

Our results indicate that at the start of therapy, MAFC and MAFMDR values, and later at 4 to 6 weeks of treatment, MAFC, MAFMRP and MAFMDR values of CD3+ cells were higher in nonresponders to biological disease modifying anti rheumatic drug (bDMARD) compared to Responders among RA patients. ROC analysis revealed that RA patients with MAFC values above 21.3 in CD3+ cells at the start of bDMARD therapy are likely to be nonresponders.

While MDR1 expression on healthy CD4+ and CD19+ lymphocytes is only marginal, significant upregulation was demonstrated in RA patients [84,85] as a result of the presence of danger-associated molecular patterns (DAMPs). Furthermore, the expression level of MDR1 was significantly elevated in methotrexate nonresponder patients compared to responders. The YB-1 transcriptional factor may have an essential role in the regulation of MDR1 in lymphocytes of RA patients upon translocation from the cytoplasm into the nucleus. Inflammation-derived TNF-a appears to play a crucial role in this phenomenon [86].

While the etiology of RA remains unknown, inflammatory mediators appear to drive the evolution of the disease. In particular, TNF-α together with proinflammatory cytokines, including IL-1β and IL-6, have been shown to be pivotal in promoting cytokine, chemokine and matrix metalloproteinase production within the RA synovium, along with cellular activation and joint erosion [137].

As a response for various signals (i.a. TNF-α) S1P is generated and exported out of the synoviocytes by ABC transporters to act on the same cell (autocrine effect) or on nearby cells (paracrine effect). S1P engages with its receptors (S1P1-3) to mediate a diverse array of signaling pathways, impacting fundamental biological processes that are integral to the pathogenesis of RA, such as cell proliferation and survival, cell migration, and inflammatory mediator secretion. [137].

As visualized in Figure 1. important inflammation mediators such as PAF, S1P, leucotrienes and prostanoids are MDR—ABC transporter victims thus measuring the activity of these transporters could result useful information on the inflammation process.

In an earlier study, Rhodamine 123 was used as a substrate of MDR1 and verapamil as transporter inhibitor [43]. Flow cytometry based analysis did not find any correlations between methotrexate responders and nonresponders at baseline, however in nonresponders the functional activity was upregulated 4 months following therapy.

In a more recent study, the activity of MDR1 and MRP1 was investigated on different leukocyte subsets, namely granulocytes, monocytes, lymphocytes, CD4+, CD8+ and CD19+ cells from RA patients and controls (traumatic injury patients and healthy volunteers, respectively). Based on DAS28 scores, RA patients were categorized into methotrexate responder and nonresponder groups. Since side effects easily develop during methotrexate treatment, an additional methotrexate intolerant group was generated with intolerable side effects. In case of granulocytes, the functional activity of MRP1 was significantly higher in methotrexate responders vs. methotrexate nonresponders highlighting the complexity of the issue. Furthermore, even higher functional activity was demonstrated in methotrexate intolerant individuals in comparison with methotrexate responders. Data suggest that MDR1 and MRP1 functional activity does not seem to affect the response rate to methotrexate therapy of RA patients, but it might be useful in predicting MTX-side effects. Therefore, the authors concluded that determining MAF values might be useful in predicting methotrexate intolerance in order to avoid harmful side effects of methotrexate therapy [76].

Although the role of MDR—ABC transporter activity in the prediction of response to methotrexate has been characterized in RA [128 129], little is known about the relation of MDR proteins to therapeutic success of biologicals. In contrast to methotrexate and other conventional synthetic disease modifying anti rheumatic drugs (csDMARDs), these molecules do not enter the cell, and are therefore not substrates of MDR proteins. However, the cytokines they target may indirectly interact with these transporters [81,82, 130]. For instance, a recent study described that stimulation with TNF-a induced MDR1 and MRP1 expression via NF-kB signaling in astrocytes [131]. Therefore, we hypothesized that MDR activity may be used as a biomarker to predict therapeutic success in RA. A similar crosstalk in lymphocytes could provide the molecular basis of the findings of our clinical study, yet to be confirmed in future investigations. Induction of MDR—ABC transporters by IL-17 have been [139] noted. As anti-IL-17 therapy in inflammatory diseases is [132] increasing. Therefore, a similar scenario can be envisioned in rheumatoid arthritis [133].

Our ROC analysis revealed that the assessment of multidrug activity of peripheral blood lymphocytes carries predictive value for response to bDMARD treatment in RA patients at the start of therapy. Patients with MAF values above the cut-off thresholds are likely to be nonresponders to treatment. Of note, these cut-off values are all below the respective reference ranges in healthy individuals established in our earlier study.

Although baseline MAF values before the start of bDMARD therapy did not differ between healthy controls and the RA patient groups, such differences were already present between responder and nonresponder RA patients in case of T cells. Future studies may be able to explore whether differences in MAF values are present in therapy naïve RA patients compared to healthy controls as well as the changes in the activity of the studied transporters over the course of csDMARD treatment until reaching the need for bDMARD therapy. This information could provide more insights into the pathophysiological role of these transporters and may enable even earlier prediction of RA treatment response.

Interestingly, no significant changes of MAF values within the respective RA patient groups were demonstrated with the progress of treatment (Figure 8). However, considerable intra- and inter-patient variations were observed within both patient groups in these values. This observation may be related to limitations of our study, namely the relatively small number of patients included in each group and in particular the heterogeneity of the bDMARD treatment received. A larger number of patients in future studies will allow to create and compare homogenous patient groups in terms of the therapy applied.

In conclusion, our results indicate that the determination of MAFC values in CD3+ cells of RA patients may be of predictive value prior to the initiation of biological therapy to establish whether the patient will demonstrate sufficient therapeutic response. Measuring MAFC, MAFMRP and MAFMDR values in CD3+ cells at 4 to 6 weeks after the start of treatment further improves the accuracy of prediction as to whether adequate therapeutic response may be expected.

5.1. Novel results of the thesis

I. Reference MAF values were established on CD3+ lymphocytes in a normal population, providing a baseline to compare the pathological transporter activity in diseases.
II. MAF MDR1, and its derivative MAFC decrease with age. It must be considered when MDR1 substrate drugs are administered.
III. No gender dependence was found for any MAF values.
IV. At baseline MAFC and MAFMDR values, on CD3+ cells, are higher in nonresponders to anti rheumatic biological therapy compared to responders. It may be of predictive value before the initiation of biological treatment.
V. During the biological treatment at 4 to 6 weeks MAFC, MAF MRP and MAF MDR values of CD3+ cells and MAF MRP values of CD19+ were higher in nonresponders to anti rheumatic biological therapy compared to responders. It may be of predictive value during biological treatment.

6. Summary

MDR—ABC transporters are important biomarkers of drug resistance in cancer and in autoimmune conditions and are increasingly used in clinical diagnosis and the prediction of therapy efficacy in these conditions. We established the activity of the three clinically most relevant MDR transporters (MDR1, MRP1 and BCRP) in CD3+ lymphocytes using a novel flow cytometry based method from 120 healthy volunteers in order to describe normal reference values of the activity of these transporters. The effects of gender and age were also determined.

Using the normal reference values in a second study we determined the predictive value of MDR1, MRP1 and BCRP activity measurement for biological therapeutic response in 39 RA patients before as well as 4 to 6 and 12 weeks after the initiation of biological therapy. 35 Healthy volunteers were included as control.

The MultidrugQuant™ Kit was used for measurements. In this assay, fluorescent reporter substrates (calcein AM for MDR1 and MRP1 and mitoxantrone for BCRP, respectively) are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of specific inhibitors (verapamil for MDR1 and MRP1, indomethacin for MRP1 and KO134 for BCRP, respectively), allowing for quantitative, standardized assessment. Cell surface staining was applied

to select CD3+ cells in the normal reference value study and CD3+ and CD19+ cells in the predictive biomarker assessment study.

Informed consent was obtained from all subjects, and our study was approved by an independent ethical committee of the institutions.

MAF values of MRP1 and BCRP are independent from age. MAFC and MAF of MDR1 show negative correlation with the age of the studied subjects. No difference was detected in any of the four MAF values between men and women. Gender does not affect the presence or lack of correlation between MAF values and age.

At the start of therapy, MAFC (composite MAF of MRP1 and MDR1) and MAFMDR values and at 4 to 6 weeks of treatment, MAFC, MAFMRP and MAFMDR values of CD3+ cells can be used to predict unfavorable biological therapeutic response in RA

The determination of the functional activity of MDR-ABC transporters is achievable using a flow cytometry based standardized method. Having established the normal range of MAF values of a healthy population, and determined the cut-off thresholds for MAF values between biological therapy responders and nonresponders our results allow for the development of novel flow cytometry based diagnostic algorithms in rheumatoid arthritis.

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10. Annex

I

Cytometry Part B (Clinical Cytometry) 96B:469–474 (2019)

Original Article

Determination of Reference Values of MDR-ABC Transporter Activities in CD3+ Lymphocytes of Healthy Volunteers Using a Flow Cytometry Based Method

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Background: MDR transporters are important biomarkers of drug resistance in cancer and in autoimmune conditions. We determined the MDR1, MRP1 and BCRP activity in CD3+ lymphocytes using a flow cytometry based method from 120 healthy volunteers in order to describe normal reference values of the activity of these transporters. The effects of gender and age were also determined.

Methods: The Solvo MDQ Kit™ was used for measurements. In this assay, fluorescent reporter substrates (Calcein-AM for MDR1 and MRP1 and mitoxantrone for BCRP, respectively) are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of specific inhibitors (verapamil for MDR1 and MRP1, indomethacin for MRP1 and KO134 for BCRP, respectively), allowing for quantitative, standardized assessment. Cell surface staining was applied to select CD3+ cells.

Results: MAF values of MRP1 and BCRP are independent from age. MAFC and MAF of MDR1 show negative correlation with the age of the studied subjects ($P = 0.003$, $r = -0.27$ and $P = 0.0001$, $r = -0.34$, respectively). No difference was detected in any of the four MAF values between men and women. Gender does not affect the presence or lack of correlation between MAF values and age.

Conclusions: The determination of the functional activity of MDR-ABC transporters is achievable using a flow cytometry based standardized method. Having established the normal range of MAF values on CD3+ lymphocytes of a healthy population, our results allow for the development of novel flow cytometry based diagnostic tools.

Key terms: BCRP; Calcein; MDR1; mitoxantrone; MRP1

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INTRODUCTION

Multidrug resistance (MDR-ABC) transporters (MDR1/P-gp/ABCB1; MRP1/ABCC1; BCRP/ABCG2) transport a variety of endobiotics (1) as well as drugs (2) and are important biomarkers of drug resistance in cancer (3) and in autoimmune conditions, such as rheumatoid arthritis (RA) (4). $CD3^+$ T lymphocytes play an important role in the regulation of the immune response under physiological conditions, as well as

Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; MAF, MDR activity factor; MDR, multidrug resistance; MRP, multidrug resistance; RA, rheumatoid arthritis

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anti-tumor immunity. Their deranged function and interaction with autoantigens is a cornerstone in the development of autoimmunity and the pathogenesis of RA and other autoimmune disorders (5).

The qRT-PCR, immunohistochemistry, and Western blot are the most frequently used methods to determine the MDR-ABC transporter status in clinical samples. More recently, mass spectrometry based methods have been described to quantify transporter expression (6). On the other hand, several polymorphisms affecting MDR-ABC transporter function have been reported (7,8). Therefore, relevance of even protein levels as solitary pieces of data is questionable. Some of the genetic variants affect transporter trafficking and, thus, FACS-based determination of cell surface expression of MDR-ABC transporters is a significant progress (9). However, antibodies recognizing the extracellular MDR1 (10,11) and BCRP (12) epitopes are conformation sensitive, making their determination challenging.

Functional laboratory tests are reasonable alternatives for the determination of transporter activity in cell suspensions. These tests utilize fluorescent molecules that can penetrate the cell membrane and once in the cytosol, they serve as specific substrates of the transporter of interest. Rhodamine 123 (13,14) and Calcein-AM (15–17) are the most frequently used fluorophores for MDR1. Calcein-AM is also a substrate of MRP1 (18) and using specific inhibitors of the transporters, MDR1 and MRP1 activity can be simultaneously determined (17). BCRP has several fluorescent substrates and both the Hoechst dye (Hoechst 33342) (19) and mitoxantrone (20) have been used in FACS-based assays.

Transporter expression (21) and function (22) have been shown to depend on gender in preclinical specimens. Gender dependent expression of MDR1 (23) and BCRP (6) in human liver samples has not been confirmed. However, effect of gender may be tissue specific (24), therefore, should be considered.

Age is another covariate as decreased MDR1 function in the blood–brain barrier (BBB) has been found with aging in two independent clinical PET studies (25,26). Interestingly, no effect of aging was shown on MDR1 protein expression in hepatocytes (23). This apparent discrepancy may again underline the importance of tissue specific functional studies.

In this clinical study, we determined the MDR1, MRP1, and BCRP activity in CD3+ lymphocytes from healthy volunteers in order to describe normal reference values of the activity of these transporters. The effects of gender and age were also determined.

MATERIALS AND METHODS

Patient Recruitment

The reference MDR activity factor (MAF) intervals were determined according to the CLSI guideline C28-A2 (27) on CD3+ lymphocytes of a reference population of 120 healthy volunteers. The study protocol and the written informed consent form had ethical approval from the Medical Research Council Ethics Committee for Clinical Research (ETT TUKEB) of Hungary. The study adhered to the tenets of the most recent revision of the Declaration of Helsinki. Based on normal values of full blood count, the CD4/CD8 ratio, liver and kidney function tests, 120 healthy subjects aged 18–74 years were selected. The age distribution was 18–39 years for 49 subjects, 40–59 years for 45 subjects, and 60–74 years for 26 subjects. The gender distribution was 58 female and 62 male subjects. There was no observed adverse event or side effect in this study.

Peripheral Blood Mononuclear Cell (PBMC) Isolation

About 3 mL uncoagulated and 6 mL K_3EDTA anticoagulated peripheral blood samples were collected at the time of examination from volunteers. Heparin is known to interfere with the activity of MDR protein; therefore, the use of heparinized blood is not recommended (28). PBMCs were separated by density gradient centrifugation using Ficoll Histopaque-1077 (Cat. No: H8889, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Since this is an ATP-dependent functional assay, it requires cells in good condition not depleted of intracellular energy stores. Therefore, blood samples were processed within 6 h following sampling and stored at room temperature before processing.

Flow Cytometry

Measurements were conducted on a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) equipped with 488 nm argon and 635 nm red diode lasers. The equipment was calibrated with BD Calibrite 3 beads (Cat. No: 340486, BD Biosciences).

The Solvo MDQ Kit™ (Catalogue Number: MDQ101D) was used strictly following the manufacturer's instructions. Separated PBMCs were washed twice with 5 mL of HBSS by centrifugation at 300*g* for 10 min. Supernatants were discarded and cells were counted. A cell suspension containing 2–5 × 10^6 cells was prepared using HBSS and 800 μL of cell suspension was added into 15 tubes.

For measuring the activity of MDR1 and MRP1 (tubes 1–9), 5 μL of verapamil (MDR1 and MRP1 inhibitor) was added in tubes 1–3, 5 μL of indomethacin (MRP1 inhibitor) in tubes 4–6, and 5 μL of HBSS was added in tubes 7–9. Samples were incubated in 37°C for 5 min. About 200 μL of calcein-AM was added in tubes 1–9. Samples were incubated for 10 min at 37°C. Samples were centrifuged for 1 min at 2,000*g*. Supernatants were discarded and cells were resuspended in 500 μL of HBSS. Anti-human CD3-PerCP monoclonal antibody (Cat. No: 345766, BD Biosciences) was applied for cell surface staining according to the manufacturer's instructions. Cells were centrifuged at 2,000*g* for 1 min. Supernatant was discarded and cells were resuspended

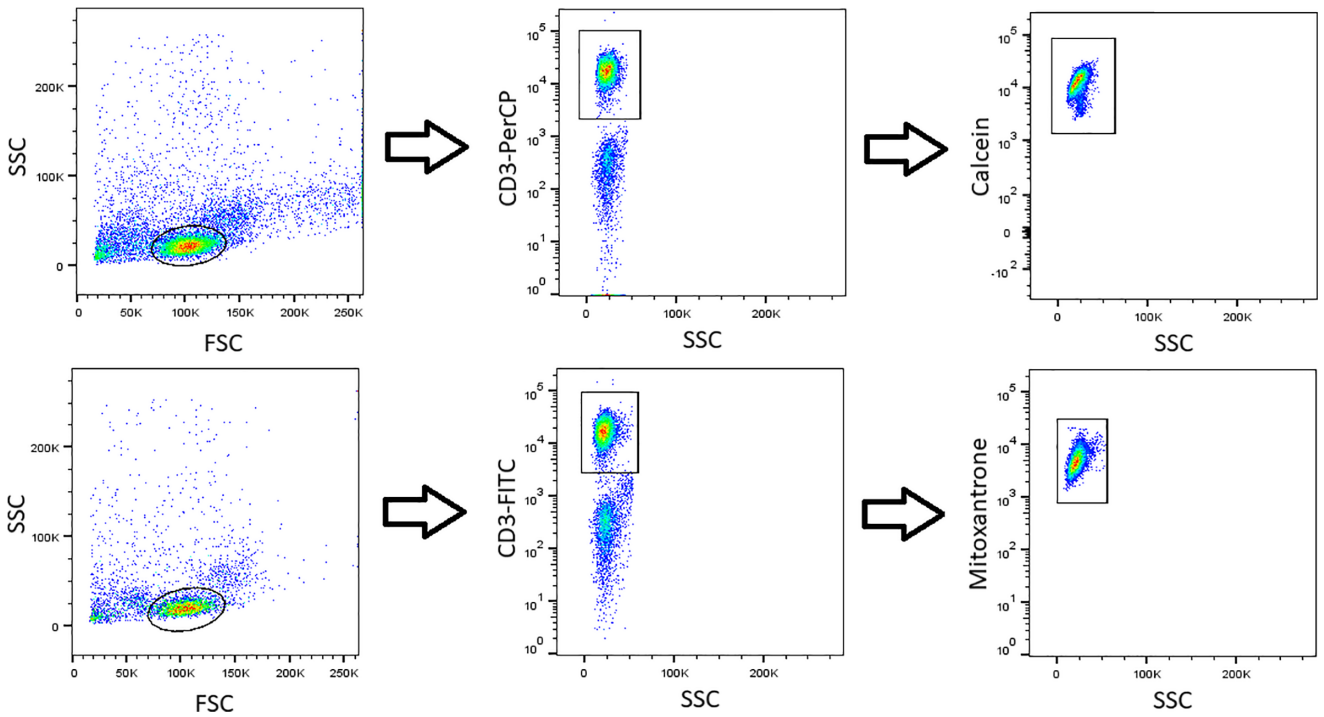

FIG. 1. Gating strategy to determine Calcein and mitoxantrone fluorescence on a representative sample. FSC, forward scatter characteristics; SSC, side scatter characteristics. [Color figure can be viewed at wileyonlinelibrary.com]

in 500 μL of HBSS and run on the flow cytometer immediately.

For measuring the activity of BCRP (tubes 10–15), 5 μL of KO134 (BCRP inhibitor) was added into tubes 10–12 and 5 μL of HBSS was added into tubes 13–15. Samples were incubated for 5 min at 37°C. About 5 μL of mitoxantrone was added into tubes 10–15 and samples were incubated for 30 min at 37°C. Cells were centrifuged for 1 min at 2,000*g*. Supernatant was discarded and cells were resuspended in 500 μL of HBSS. Anti-human CD3-FITC monoclonal antibody (Cat. No: 345763, BD Biosciences) was applied for cell surface staining according to the manufacturer's instructions. Cells were centrifuged at 2,000*g* for 1 min. Supernatant was discarded and cells were resuspended in 500 μL of HBSS and run on the flow cytometer immediately.

As seen, measurements were performed in three technical replicates. CVs of technical replicates were between 0.6% and 4.1%. MAF values were calculated using medians of geometric mean fluorescent intensities (MFIs) of the replicates as the difference between MFIs of cells with and without the specific inhibitors, respectively. The gating strategy applied is demonstrated on a representative sample in Figure 1.

$$\text{MAFC} = 100 \times (F_{\max} - F_o)/F_{\max}$$

$$\text{MAF of MRP1} = 100 \times (F_{\text{MRP1}} - F_o)/F_{\max}$$

$$\text{MAF of MDR1} = \text{MAFC} - \text{MAF of MRP1}$$

$$\text{MAF of BCRP} = 100 \times (F_{\text{MX}} - F_o)/F_{\text{MX}}$$

$F_{\max}/F_{\text{MX}}$: Calcein/mitoxantrone fluorescence with verapamil or KO134, respectively

F_o : fluorescence without inhibitor

F_{MRP1} : Calcein fluorescence with indomethacin

Statistics

The distribution of MAFC and MAF of MDR1 values are acceptable as normal according to the Shapiro–Wilk test ($P > 0.05$) with no outlier data (Grubbs test, $P > 0.05$). For MAF_MRP1 values the distribution appears non-normal (Shapiro–Wilk test, $P < 0.05$) but there are no outlier values (Grubbs test, $P > 0.05$). MAF_BCRP values also show non-normal distribution with three outlier values. The deviation from the normal distribution can be explained partly by the multiple "0" values obtained for MAF.

We applied univariate tests of significance to correlate MAF values with the age of the studied subjects using Sigma-restricted parameterization. Because the gender distribution of MAF values was normal, Student's *t*-tests, and *F*-tests were used for comparisons between male and female subjects.

RESULTS

Activities of multidrug transporters are reflected by the difference between the amount of Calcein/mitoxantrone accumulated in the presence or absence of the

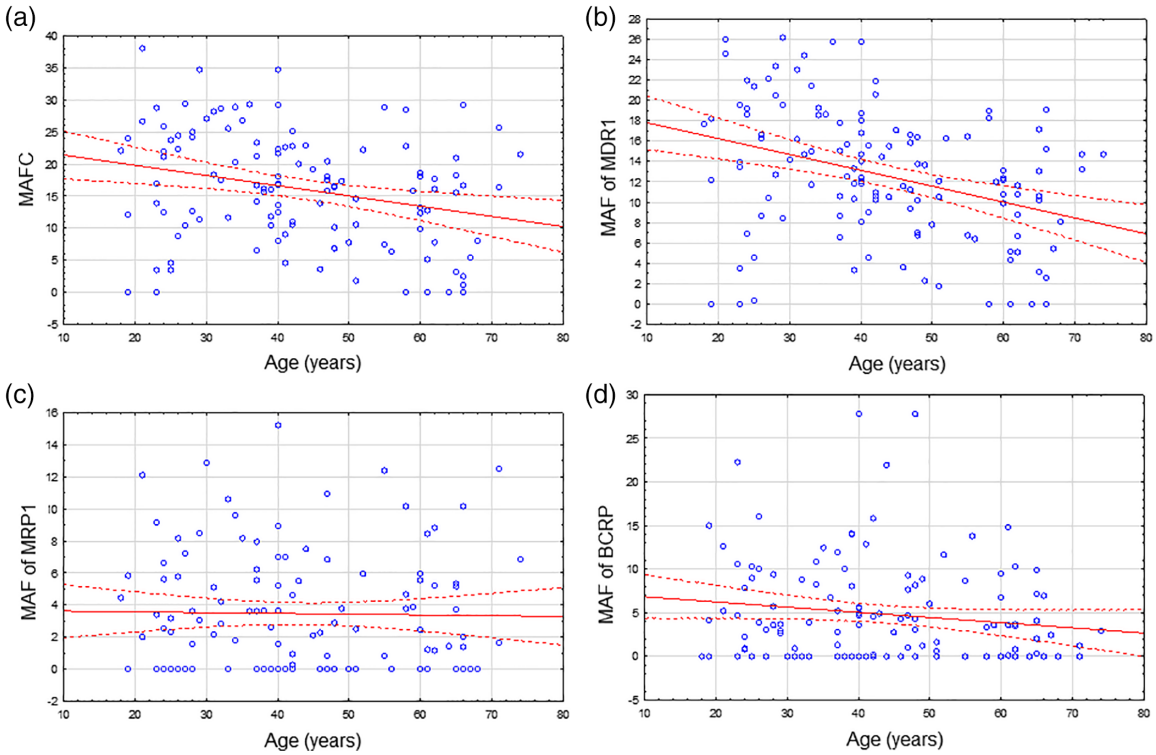

FIG. 2. Scatterplots of the MAFC value (a) and MAF values of MDR1 (b), MRP1 (c) and BCRP (d) against age. 95% confidence interval is represented in red. MAFC and MAF of MDR1 show negative correlation with the age of the studied subjects. [Color figure can be viewed at wileyonlinelibrary.com]

selective inhibitor(s). When calculating the MAF values, this accumulation difference is normalized to the dye uptake measured in the presence of the inhibitor. Thus, the result of the test becomes independent from factors influencing the cellular accumulation of Calcein other than the activity of the multidrug transporters. MAFC is a composite activity value for the MDR1 and MRP1 transporters.

Our results indicate that MAF value of MRP1 is 2.5 [0.0–12.5] (median [2.5–97.5 percentiles]) and are independent from age (Fig. 2c). MAF value of BCRP is 3.4 [0.0–22.0] (median [2.5–97.5 percentiles]) and is also independent from age (Fig. 2d). On the other hand, MAFC and MAF of MDR1 show negative correlation with the age of the studied subjects ($P = 0.003$, $r = -0.27$ and $P = 0.0001$, $r = -0.34$, respectively) (Fig. 2a and b). MAF value of MDR1 is 12.9 [0.0–25.7] (median [2.5–97.5 percentiles]) and that of MAFC is 16.5 [0.0–32.0] (median [2.5–97.5 percentiles]). MDR1 activity greatly contributes to the MAFC value and is therefore likely to be accountable for its similar correlation with age.

No difference was detected in any of the four MAF values between men and women (Table 1). Gender does not affect the presence or lack of correlation between MAF values and age (Fig. 3).

DISCUSSION

In the Solvo MDQ Kit™ assay, fluorescent reporter substrates are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of specific inhibitors. This novel method offers a standardized approach to measure MAF of the three clinically most relevant MDR transporters (MDR1, MRP1, and BCRP). Although it is primarily suitable for the determination of MAF values of peripheral blood or

Table 1
Gender-Specific MAF Values of the Investigated MDR-ABC Transporters on CD3+ Lymphocytes

	Men ($n = 62$)			Women ($n = 58$)		
	Median	2.5%	97.5%	Median	2.5%	97.5%
MAFC	16.4	1.2	29.4	16.6	0.0	34.7
MAF of MDR1	13.2	0.0	25.7	12.2	0.0	26.0
MAF of MRP1	3.0	0.0	10.2	2.1	0.0	12.9
MAF of BCRP	4.2	0.0	27.8	2.7	0.0	14.8

Data are presented as median [2.5–97.5 percentiles]. No statistically significant difference was observed in any of the investigated MAF values between men and women.

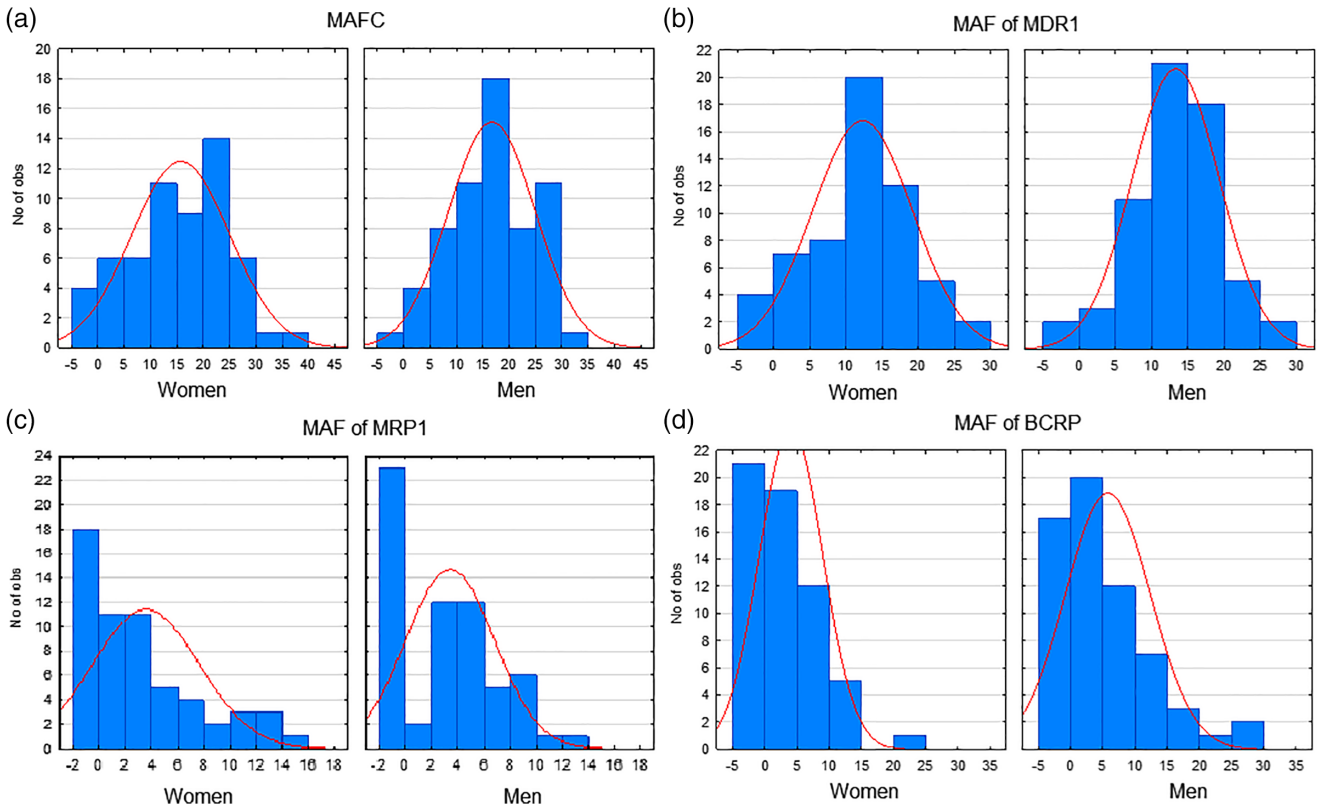

FIG. 3. Histograms representing the distribution of the MAFC value (a) and MAF values of MDR1 (b), MRP1 (c), and BCRP (d) in men and women. Gender does not affect the presence or lack of correlation between MAF values and age. [Color figure can be viewed at wileyonlinelibrary.com]

bone marrow cells on the flow cytometry platform, any cell types that are stable in a suspension could be used for measurements. However, taking into account the easy accessibility of peripheral blood from patients, the clinically most relevant utilization of this method may be hemato-oncology and immunology. The calculation of MAF values is independent from variability caused by inter-assay, equipment or lab environment factors, as detailed in the Methods section and demonstrated by preliminary experiments (data not presented). A further great benefit of the method is the flexibility regarding the number of cell types analyzed depending on the fluorescent cell surface markers used, offering a comprehensive approach for the assessment of transporter activity in multiple clinically relevant cell subsets at the same time.

MAF of MDR1, and consequently the MAFC value, which consists of the functional activity of both MDR1 and MRP1, decrease with age. While this has not been demonstrated earlier in T lymphocytes, decreased MDR1 function with aging in the BBB has been described by both van Assema et al. and Bauer et al. verified by PET studies (25,26). On the contrary, no effect of aging was shown on MDR1 protein expression in hepatocytes (23). These findings demonstrate the importance of tissue specificity regarding MDR1 functionality, potentially affecting the design of clinical trials, as well as diagnosis and treatment of patients throughout different age groups. Drugs that are substrates of the MDR1 protein may therefore need to be administered in different doses depending on age to achieve the same therapeutic effect.

A potential extension of our current study may be the determination of MAF values of the investigated transporters, as well as their age and gender dependence in other cell and tissue types, especially those responsible for drug metabolism, such as hepatocytes and renal cells.

In contrast with age, no effect of gender was noted on the activity of the studied transporters, as MAF values of MDR1, MRP1, and BCRP, as well as the MAFC value are consistent across both male and female subjects.

In conclusion, our findings demonstrate that the determination of the functional activity of MDR-ABC transporters is achievable using a flow cytometry based standardized method and complements other investigation modalities, such as expression levels of the transporters. Having established the normal range of MAF values on CD3+ lymphocytes of a healthy population, our results enables investigators to study the functional activity of MAF in different disease states, such as leukemia or autoimmune disorders, allowing for the development of novel flow cytometry based diagnostic tools.

In the future, these tools may help to improve disease activity and therapeutic response monitoring in the clinical setting.

CONFLICT OF INTEREST

PS, AA, KF and GT are/were employed by MDQuest Ltd. KJ, ZH, JMZ and PK are/were employed by SOLVO Biotechnology. SB, JK and SS received honoraria from SOLVO Biotechnology.

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II

Peripheral Lymphocyte Multidrug Resistance Activity as a Predictive Tool of Biological Therapeutic Response in Rheumatoid Arthritis

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Peripheral lymphocyte multidrug resistance activity as a predictive tool of biological therapeutic response in rheumatoid arthritis

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Conflict of interest:

GT, PS, AA and KF are/were employed by MDQuest Ltd. PB, SB and ZS received honoraria from MDQuest Ltd.

Running head: MDR activity in RA

ABSTRACT

Objective: Multidrug resistance (MDR) transporters may be used as biomarkers to monitor disease progression in RA and as a predictive tool to establish responsiveness to biological therapy. In this multicenter clinical trial, we aimed to assess the predictive value of MDR1, MRP1 and BCRP activity measurement for biological therapeutic response in RA before as well as 4 to 6 and 12 weeks after the initiation of biological therapy.

Methods: Peripheral blood samples were collected from 27 bDMARD Responders and 12 Non-responders at the indicated time points as well as from 35 healthy controls. MDR activity (MAF) of MDR1, MRP1 and BCRP was measured in CD3+ and CD19+ cells using the Solvo MDQ Kit™ and cell surface staining by flow cytometry following PBMC isolation.

Results: At the start of therapy, MAFC (composite MAF of MRP1 and MDR1) and MAFMDR values and at 4 to 6 weeks of treatment, MAFC, MAFMRP and MAFMDR values of CD3 cells were higher in Non-responders compared to Responders. ROC analysis revealed that RA patients with MAFC values above 21.3 in CD3 cells at the start of bDMARD therapy are likely to be Non-responders. At 4 to 6 weeks of treatment, MAFC values above 20.3, MAFMRP values above 6.0 and MAFMDR values above 13.9 in CD3 cells also predict unfavorable response.

Conclusions: Our results indicate that the determination of MAFC values in CD3 cells of RA patients may be of predictive value prior to the initiation of biological therapy to establish whether the patient will demonstrate sufficient therapeutic response.

KEYWORDS

BCRP, bDMARD, MDR1, MRP1, multidrug resistance, T cells, therapeutic response

INTRODUCTION

Rheumatoid arthritis (RA) affects approximately 0.5-1% of the population and causes chronic synovial inflammation eventually leading to joint destruction and disability [1]. Early diagnosis and immediate, effective therapy are crucial in order to prevent joint deterioration, functional disability and unfavorable disease outcome. The optimal management of RA is needed within 3-6 months after the onset of disease, therefore a very narrow „window of opportunity" is present to achieve remission or at least low disease activity (LDA) [2,3]. Therefore, it is very important to predict the efficacy of expensive biologicals at early stages of treatment. Although a new generation of drugs is available, there are no validated circulating biomarkers of prognostic use or to predict response to specific therapies [4].

Multidrug resistance (MDR-ABC) transporters (MDR1/P-gp/ABCB1; MRP1/ABCC1; BCRP/ABCG2) are important components in the development of drug resistance in malignancies [5] and in autoimmune conditions, such as RA [6]. Although studies of the crystal structure and function of MDR-ABC transporters suggest that they are not directly involved in the release of cytokines and chemokines, they may extrude other intracellular small molecules influencing the inflammatory balance. Thus they may play an important role in the pathogenesis of RA via influencing cell migration, proliferation and inflammation in an indirect manner. Therefore, MDR-ABC transporters may also be important biomarkers of disease progression in RA. The assessment of MDR protein activity may help physicians to evaluate how patients will respond to biological treatment and may support the decision whether there is a necessity to modify the treatment.

The most important csDMARDs (including methotrexate, sulfasalazine, leflunomide and hydroxychloroquine) are substrates of MDR proteins. For this reason, MDR activity of RA patients on csDMARD therapy has been extensively studied and the expression, polymorphisms and activity of MDR proteins has been linked to therapeutic success of csDMARDs, especially that of methotrexate. However, little is known about the relation of MDR proteins to therapeutic success of biologicals, such as anti-TNF agents. Although these molecules do not enter the cell, and are therefore not substrates of MDR proteins, the endobiotics, such as the cytokines they target are known to interact with these transporters [7-9]. Through influencing the distribution of cytokines and other soluble factors within the cell and in its most proximal environment, the function of T- and B-cells may be affected by the activity of the transporters, balancing the effect of such factors on lymphocyte activation, proliferation, production of other cytokines and antibodies, etc. Therefore, MDR activity may be used as a biomarker of therapeutic success in RA and other autoimmune disorders.

In this multicenter clinical trial, we aimed to assess the predictive value of flow-cytometry based multidrug resistance activity measurement for biological therapeutic response in rheumatoid arthritis. We aimed to assess the activity of three clinically relevant MDR proteins (MDR1, MRP1, BCRP) in CD3+ and CD19+ lymphocytes of RA patients before as well as 4 to 6 and 12 weeks after the initiation of biological therapy.

METHODS

Patient Recruitment

39 RA patients were recruited at the outpatient clinics of the Department of Rheumatology, University of Debrecen, Hungary and the Department of Rheumatology and Clinical Immunology, Charité, Berlin, Germany. Patients were sampled before the start of biological treatment as well as between 4 and 6 weeks and at 12 weeks of treatment. DAS28 and CRP values were also recorded at this time. Patients were regarded as non-responders (n = 12) if DAS28 values showed a decrease of less than 25% between the start of biologicals and at 12 weeks of treatment (arbitrary cut-off). Patient characteristics as well as details of the therapy received are included in Table 1.

Healthy controls (n = 35) were sampled at the Department of Rheumatology, University of Debrecen, Hungary on a single occasion. They had a negative history of autoimmune disorders including RA and a negative status upon physical examination as well as no infectious symptoms within three weeks before sampling.

Exclusion criteria for all participants included chronic infectious diseases requiring systemic treatment, autoimmune diseases other than RA, immunodeficiencies, allergic diseases and hematological malignancies or solid tumors.

Written informed consent was obtained from all participants and the study adhered to the tenets of the most recent revision of the Declaration of Helsinki. Ethical approval for the study was granted by local ethics committees of the University of Debrecen (TUKEB 21018/2014/EKU) and Charité (EA1/193/10), respectively.

Peripheral Blood Mononuclear Cell (PBMC) isolation

6 mls of EDTA anticoagulated peripheral blood sample was collected. PBMCs were separated by density gradient centrifugation using Ficoll Histopaque-1077 (Cat. No: H8889, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Flow Cytometry

Measurements were conducted on a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) equipped with 488 nm and 635 nm lasers or on a Miltenyi MACSQuant flow cytometer, equipped with 405nm, 488 nm and 638 nm lasers, respectively.

The Solvo MDQ Kit™ was used strictly following the manufacturer's instructions. In this assay, fluorescent reporter substrates are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of highly selective inhibitors, allowing for quantitative, standardized assessment. PBMCs were loaded with fluorescent MDR activity reporter substrates (1.25 uM working solution of Calcein-AM for MDR1 and MRP1, em: 515 nm, incubated for exactly 10 minutes followed by rapid centrifugation and 1 mM working solution of mitoxantrone for BCRP, em: 684 nm, respectively, incubated for exactly 30 minutes followed by rapid centrifugation) and treated with highly selective MDR protein inhibitors (12.7 mM working solution of verapamil for MDR1 and MRP1, 2 mM working solution of indomethacin for MRP1 and 0.1 mM working solution of KO134 for BCRP,

respectively) to obtain multidrug activity factor (MAF) values (Figure 1). Note that the above working concentrations are further diluted upon addition to the cell suspension to avoid cell toxicity as described in the manufacturer's instructions.

Cell surface staining was applied to select CD3+ and CD19+ cells using anti-human CD3-PerCP and CD19-PE monoclonal antibodies (Cat. No: 345766 and 345789, respectively, both BD Biosciences) in case of Calcein-AM stained cells and anti-human CD3-FITC and CD19-PE monoclonal antibodies (Cat. No: 345764 and 345789, respectively, both BD Biosciences) in case of mitoxantrone stained cells according to the manufacturer's instructions. Samples were run on a flow cytometer immediately following cell surface staining.

Activities of multidrug transporters are reflected by the difference between the amount of Calcein/mitoxantrone accumulated in the presence or absence of the selective inhibitor(s). When calculating the MAF values, this accumulation difference is normalized to the dye uptake measured in the presence of the inhibitor. Thus, the result of the test becomes independent from factors influencing the cellular accumulation of Calcein/mitoxantrone other than the activity of the multidrug transporters. The inter-assay variability of the test is CV <10%.

MAF values were calculated from the difference between the geometric mean fluorescent intensity (MFI) of cells with and without the highly selective inhibitors, respectively.

MAFC (composite MAF of MRP1 and MDR1) = 100 x (Fmax – Fo) / Fmax

MAF of MRP1 = 100 x (FMRP1 – Fo) / Fmax

MAF of MDR1 = MAFC – MAF of MRP1

MAF of BCRP = 100 x (FMX – F0) / FMX

Fmax/FMX: Calcein/mitoxantrone fluorescence with verapamil or KO134, respectively

Fo: fluorescence without inhibitor

FMRP1: Calcein fluorescence with indomethacin

Statistics

Comparisons were made using the Kruskall-Wallis test or the Mann-Whitney test as the distribution of data appeared to be non-normal according to the Shapiro-Wilk test. p values < 0.05 were considered significant. Statistics were calculated using the GraphPad Prism 5 software (La Jolla, CA, USA).

RESULTS

DAS28 values decreased upon treatment in Responders in contrast to Non-responders. Of note, initial DAS28 values were higher in the Responder group compared to Non-responders (Table 2, Figure 2). Neither differences were observed in CRP values between the two group, nor changes in CRP were demonstrated upon treatment.

MAF of MRP1 in CD3 cells was higher at 12wk in Non-responders compared to Controls. No other statistically significant difference was noted in MAF values between Controls and RA patients.

Control values were within the reference range established in our earlier study [10].

At the start of therapy, MAFC and MAFMDR values of CD3 cells were higher in Non-responders compared to Responders. At 6wk, MAFC, MAFMRP and MAFMDR values of CD3 cells as well as MAFMRP values of CD19 cells were higher in Non-responders compared to Responders (Table 2).

No significant changes were demonstrated in MAF values in the respective RA patient groups with the progress of treatment (Figure 3).

No difference was demonstrated in MAFBCRP values in CD3 or CD19 cells between Responders and Non-responders.

ROC analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6wk. Cut-off thresholds were calculated for MAF values with ROCs of adequate p and AUC values (Figure 4). Patients with MAF values above the respective cut-off thresholds are likely to be Non-responders to treatment (MAFC of CD3 cells at 0wk: $p = 0.043$, AUC = 0.68; MAFC of CD3 cells at 6wk: $p = 0.033$, AUC = 0.72; MAFMDR on CD3 cells at 6wk: $p = 0.048$, AUC = 0.70; MAFMRP on CD3 cells at 6wk: $p = 0.049$, AUC = 0.69).

DISCUSSION

Our results indicate that at the start of therapy, MAFC and MAFMDR values, and later at 4 to 6 weeks of treatment, MAFC, MAFMRP and MAFMDR values of CD3 cells were higher in Non-responders to bDMARD compared to Responders among RA patients. ROC analysis revealed that RA patients with MAFC values above 21.3 in CD3 cells at the start of bDMARD therapy are likely to be Non-responders.

While MDR1 expression on healthy CD4+ and CD19+ lymphocytes is only marginal, significant upregulation was demonstrated in RA patients [11,12] as a result of the presence of danger-associated molecular patterns (DAMPs). Furthermore, the expression level of MDR1 was significantly elevated in methotrexate (MTX) non-responder patients compared to responders. The YB-1 transcriptional factor may have an essential role in the regulation of MDR1 in lymphocytes of RA patients by translocation from the cytoplasm into the nucleus. Inflammation-derived TNF-a appears to play a crucial role in this phenomenon [13].

In an earlier study, Rhodamine 123 was used as a substrate of MDR1 and verapamil as transporter inhibitor [14]. Flow cytometry based analysis did not find any correlations between MTX responders and non-responders at baseline, however in non-responders the functional activity was upregulated 4 months following therapy.

In a more recent study, the activity of MDR1 and MRP1 was investigated on different leukocyte subsets, namely granulocytes, monocytes, lymphocytes, CD4+, CD8+ and CD19+ cells from RA patients and controls (traumatic injury patients and healthy volunteers, respectively). Based on DAS28 scores, RA patients fell into MTX responder and non-responder groups. Since side effects easily develop during MTX treatment, an additional MTX intolerant group was generated with intolerable side effects. In case of granulocytes, the functional activity of MRP1 was significantly higher in MTX responders vs. MTX non-responders. Furthermore, even higher functional activity was demonstrated in MTX intolerant individuals in comparison with MTX responders. Therefore, the authors concluded that determining MAF values might be useful in predicting MTX intolerance in order to avoid harmful side effects of MTX therapy [15].

Although the role of MDR transporter activity in the prediction of response to MTX has been characterized in RA, little is known about the relation of MDR proteins to therapeutic success of biologicals. In contrast to MTX and other csDMARDs, these molecules do not enter the cell, and are therefore not substrates of MDR proteins. However, the cytokines they target may indirectly interact with these transporters [7-9]. For instance, a recent study described that stimulation with TNF-a induced MDR1 and MRP1 expression via NF-kB signaling in astrocytes [16]. Therefore, we hypothesized that MDR activity may be used as a biomarker to predict therapeutic success in RA. A similar crosstalk in lymphocytes could provide the molecular basis of the findings of our clinical study, yet to be confirmed in future investigations.

Our ROC analysis revealed that the assessment of multidrug activity of peripheral blood lymphocytes carries predictive value for response to bDMARD treatment in RA patients at the start of therapy. Patients with MAF values above the cut-off thresholds are likely to be

Non-responders to treatment. Of note, these cut-off values are all below the respective reference ranges in healthy individuals established in our earlier study.

Although baseline MAF values before the start of bDMARD therapy did not differ between healthy controls and the RA patient groups, such differences were already present between Responder and Non-responder RA patients in case of T cells. Future studies may be able to explore whether differences in MAF values are present in therapy naïve RA patients compared to healthy controls as well as the changes in the activity of the studied transporters over the course of csDMARD treatment until reaching the need for bDMARD therapy. This information could provide more insight into the pathophysiological role of these transporters and may enable even earlier prediction of RA treatment response.

Interestingly, no significant changes of MAF values within the respective RA patient groups were demonstrated with the progress of treatment (Figure 3). However, considerable intra- and inter-patient variations were observed within both patient groups in these values. This observation may be related to limitations of our study, namely the relatively small number of patients included in each group and in particular the heterogeneity of the bDMARD treatment received. A larger number of patients in future studies will allow to create and compare homogenous patient groups in terms of the therapy applied.

In conclusion, our results indicate that the determination of MAFC values in CD3 cells of RA patients may be of predictive value prior to the initiation of biological therapy to establish whether the patient will demonstrate sufficient therapeutic response. Measuring MAFC, MAFMRP and MAFMDR values in CD3 cells at 4 to 6 weeks after the start of treatment further improves the accuracy of prediction as to whether adequate therapeutic response may be expected.

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
bDMARD	biological disease modifying antirheumatic drug
BCRP	breast cancer resistance protein
CRP	C-reactive protein
DAS	disease activity score
csDMARD	conventional synthetic disease modifying antirheumatic drug
MAF	MDR activity factor
MDR	multidrug resistance
MRP	multidrug resistance protein

MTX	methotrexate
PBMC	peripheral blood mononuclear cell
RA	rheumatoid arthritis

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FIGURE LEGENDS

Figure 1. The effects of highly selective inhibitors of MRP1 (indomethacin) and MRP1 + MDR1 (verapamil) on calcein substrate fluorescence intensity at high (A) and low (B) transporter activity, and the effects of the highly selective inhibitor of BCRP (KO134) on mitoxantrone substrate fluorescence intensity at high (C) and low (D) transporter activity in CD3 cells – representative samples of RA patients

Figure 2. DAS28 values at different sampling time points in Responder and Non-responder RA patients. *$p < 0.05$ vs. 0wk

Figure 3. Individual changes of MAF values on CD3 cells over time in Responder (n = 27) and Non-responder (n=12) RA patients. No significant changes were demonstrated in MAF values in the respective RA patient groups with the progress of treatment.

Figure 4. ROC analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6wk. Patients with MAF values above the respective cut-off thresholds are likely to be Non-responders to treatment. MAFC of CD3 cells at 0wk: $p = 0.043$, AUC = 0.68; MAFC of CD3 cells at 6wk: $p = 0.033$, AUC = 0.72; MAFMDR on CD3 cells at 6wk: $p = 0.048$, AUC = 0.70; MAFMRP on CD3 cells at 6wk: $p = 0.049$, AUC = 0.69

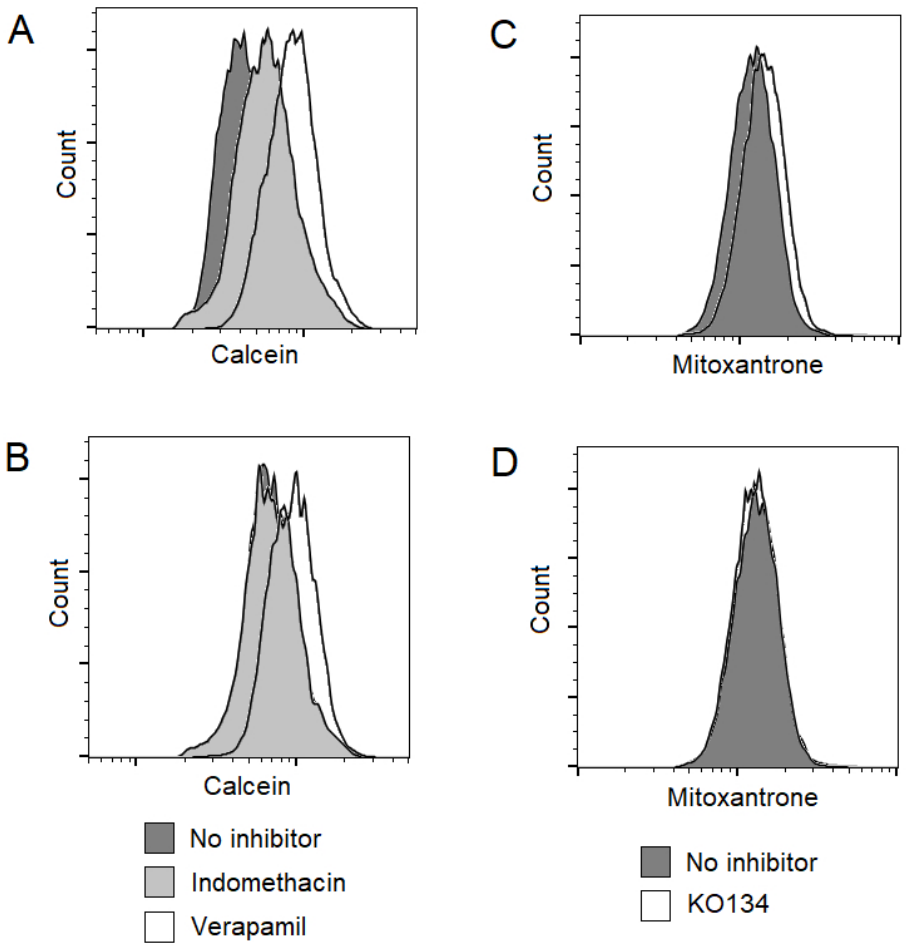

Figure 1

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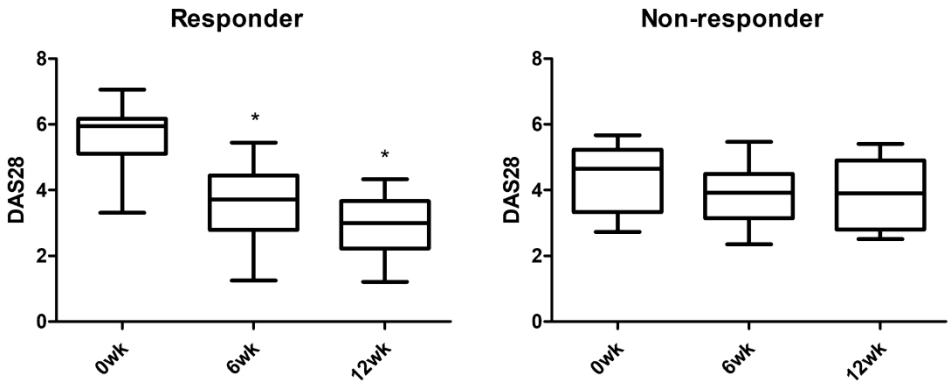

Figure 2

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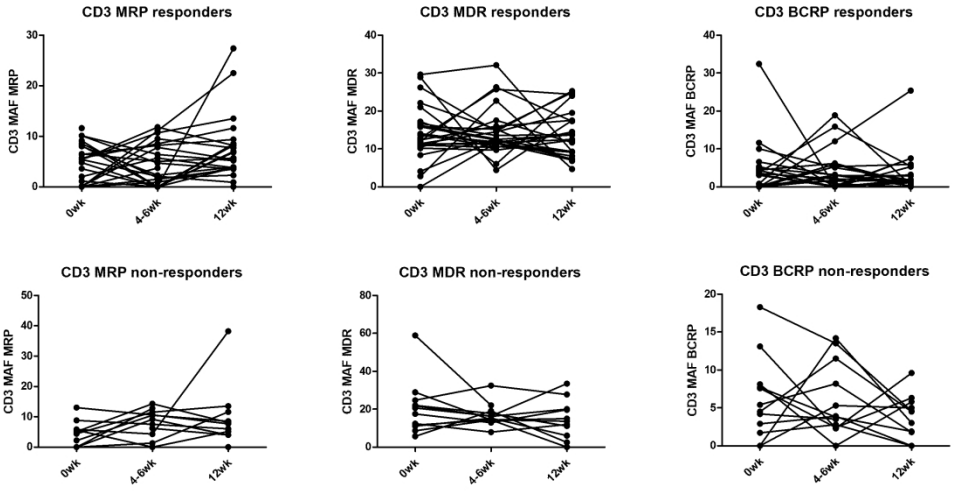

Figure 3.

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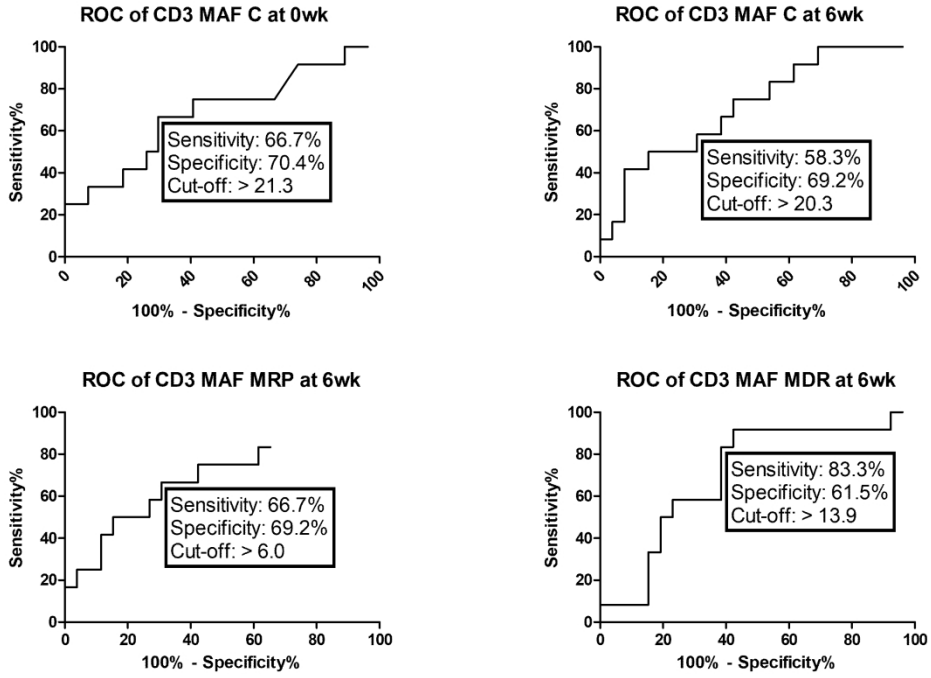

Figure 4.

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Table 1. Clinical characteristics of Responder and Non-responder RA patients as well as healthy controls. Data are expressed as median (IQR) for continuous variables and as number (percentage) for categorical variables. MTX – methotrexate

	Healthy controls (n = 35)	Responder (n = 27)	Non-responder (n = 12)
Age (years)	54 (42-62)	56 (49-61)	51 (39-61)
Gender (male/female)	4/31	2/25	1/11
RA duration (years)	-	10 (5-14)	8.5 (5-15)
No. of patients receiving MTX	-	15 (56%)	6 (50%)
No. of patients receiving prednisolone	-	9 (33%)	5 (42%)
No. of patients receiving adalimumab	-	2 (7%)	1 (8%)
No. of patients receiving certolizumab pegol	-	5 (19%)	3 (25%)
No. of patients receiving etanercept	-	7 (26%)	3 (25%)
No. of patients receiving abatacept	-	13 (48%)	5 (42%)

Table 2. Activity of various MDR transporters on CD3 and CD19 cells in RA patients and healthy controls. Data are expressed as median (IQR), $p < 0.05$ [a] vs Control, [b] vs Responder, [c] vs 0 wk value. MAFC – composite multidrug activity factor (of MRP1 and MDR1 activity), MAFMRP– multidrug activity factor of MRP1, MAFMDR – multidrug activity factor of MDR1, MAFBCRP – multidrug activity factor of BCRP

		0 wk		6 wk		12 wk	
	Control	**Responder**	**Non-responder**	**Responder**	**Non-responder**	**Responder**	**Non-responder**
DAS28	-	5.94 (5.11-6.17)	4.65[b] (3.33-5.23)	3.71[c] (2.79-4.45)	3.93 (3.14-4.50)	3.00[c] (2.23-3.67)	3.90[b] (2.81-4.90)
CRP	-	11.1 (2.6-16.6)	8.4 (1.4-15.1)	4.4 (1.3-7.9)	4.4 (1.5-10.4)	3.7 (2.1-5.6)	7.5 (2.7-11.6)
CD3 MAFC	18.3 (14.7-22.9)	18.9 (14.0-25.2)	23.5[b] (17.1-33.7)	17.1 (12.3-22.6)	22.7[b] (16.7-29.2)	18.3 (15.7-24.2)	25.2 (15.9-30.7)
CD3 MAFMRP	3.1 (1.2-5.7)	4.8 (0.0-8.0)	5.7 (2.2-8.0)	2.2 (0.0-7.9)	8.4[b] (2.1-11.3)	5.7 (3.7-8.5)	7.7[a] (4.0-11.6)
CD3 MAFMDR	14.6 (12.5-18.1)	12.9 (11.0-16.7)	19.1[b] (11.2-24.0)	12.4 (11.2-15.4)	15.8[b] (14.3-18.7)	12.5 (9.2-17.5)	13.6 (6.0-20.0)
CD3 MAFBCRP	2.5 (0.8-5.7)	3.1 (0.0-4.4)	5.0 (2.0-8.0)	2.0 (0.0-5.5)	3.9 (2.5-10.7)	1.4 (0.0-4.3)	4.5 (1.8-5.8)
CD19 MAFC	12.8 (8.9-17.9)	15.1 (8.1-22.1)	20.6 (13.5-31.0)	13.2 (9.3-20.4)	17.6 (11.4-27.2)	17.4 (13.1-22.3)	17.6 (9.2-25.9)
CD19 MAFMRP	2.2 (0.0-6.3)	0.9 (0.0-7.7)	4.4 (0.0-5.8)	0.6 (0.0-5.1)	6.8[b] (0.5-9.6)	3.2 (0.3-6.8)	5.1 (1.9-10.9)
CD19 MAFMDR	9.9 (8.0-14.0)	11.1 (6.0-16.3)	15.7 (8.4-25.4)	11.4 (5.3-14.8)	13.6 (8.6-17.7)	14.0 (7.1-17.7)	8.8 (1.9-15.7)
CD19 MAFBCRP	3.8 (1.0-6.3)	3.1 (0.7-7.0)	4.5 (0.0-11.0)	2.7 (0.0-5.2)	5.0 (3.1-8.4)	2.9 (1.3-5.1)	3.0 (1.8-3.7)

III

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— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
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(54) Title: ASSESSING RESPONSIVENESS OF RHEUMATOID ARTHRITIS PATIENTS TO BIOLOGICAL TREATMENT

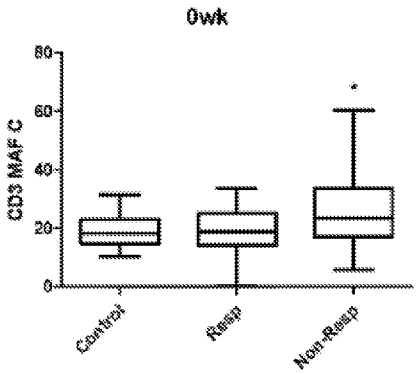

FIGURE 2.A.1

(57) Abstract: The relates to the field of diagnosis and treatment of Rheumatoid Arthritis, in particular of assessing responsiveness of rheumatoid arthritis patients to biological treatment. In particular the it has been found that measurement of MDR1 and/or MRP1 transport activities in the early phase of or before a bDMARD treatment is appropriate to provide a prediction on the effectiveness or success of bDMARD therapy once csDMARD therapy has failed. Thus, the invention relates to an in vitro diagnostic method for assessing the responsiveness of a sDMARD treated RA patient to bDMARD therapy, wherein preferably the patient is in need of a switch or modification of the sDMARD therapy by measuring transport activities of the above-mention transporters or their composite activities. The invention also relates to use of kits for the methods of the invention and methods for treatment comprising the diagnosis or prediction of the invention.

WO 2019/220158 A1

ASSESSING RESPONSIVENESS OF RHEUMATOID ARTHRITIS PATIENTS TO BIOLOGICAL TREATMENT

FIELD OF INVENTION

The invention relates to the field of diagnosis and treatment of Rheumatoid Arthritis (RA), in particular of assessing responsiveness of RA patients to biological treatment. In particular it has been found that measurement of MDR1 and/or MRP1 transport activities in the early phase of or before a biological disease modifying antirheumatic drug (bDMARD) treatment is appropriate to provide a prediction on the effectiveness or success of bDMARD therapy once classical systemic (cs)DMARD therapy has failed.

BACKGROUND OF INVENTION

Rheumatoid arthritis

RA affects approximately 0.5-1% of the population (Alamanos and Drosos 2005). The disease is more prevalent in females and it is often appears at the 5th decade of the life (Rindfleisch and Muller 2005; Kalliokoski and Niemi 2009). RA is characterized by the overactivation of the immune system and progressive joint destructions (Schett, Hayer et al. 2005). Persistent synovial inflammation finally results in joint and bone malformation (Schett, Hayer et al. 2005; Scott, Wolfe et al. 2010), that drastically cuts down the patient's quality of life (Scott, Pugner et al. 2000).

Biomarkers in RA

Although early diagnosis and immediate, effective therapy are crucial to prevent joint deterioration, functional disability and unfavourable disease outcome (Lima, Azevedo et al. 2013; Lima, Bernardes et al. 2014; Lima, Monteiro et al. 2014), a clear therapeutic target had not yet been defined. Since at the time of diagnosis the disease stage is usually severe and based on the fact that the optimal management of RA is needed within 3-6 months, therefore a very narrow "window of opportunity" is present to achieve remission or at least low disease activity (LDA)(Felson, Smolen et al. 2011; Smolen, Landewe et al. 2017). Therefore, it is very important to predict the efficacy of expensive biologicals at early stage of treatment. Although new generation of drugs is available, there are no validated biomarkers of prognostic use or to predict response to specific therapies (Verheul, Fearon et al. 2015). Although several candidate biomarkers have been investigated, their use is limited either because they require synovial sampling or rely on clinical questionnaires and symptoms besides biomarkers that cannot be objectively measured and validated. For example, a treatment algorithm based on the measurement of serum MRP8/14 levels together with clinical predictors suggested that this may have predictive potential, although this approach was not validated (Wijbrandts and Tak 2017).

Present treatment options in RA

In accordance with the current guidelines, methotrexate (MTX) therapy should be started as soon as possible (Smolen, Landewe et al. 2017), except some special cases (Wollenhaupt, Albrecht et al. 2013; Cardiel, Diaz-Borjon et al. 2014; Brenol, Nava et al. 2015; Lau, Chia et al. 2015). Based on the fact that MTX is pivotal in the maintenance of remission, the strict adherence to this particular drug is essential, however, sometimes impossible, since MTX treatment causes serious side effects, which mainly affect the gastrointestinal tract. If there is a known contraindication for MTX, leflunomide or sulfasalazine

should be the first-line treatment choice. Three months after starting therapy, a checkup visit should be performed. If the remission was reached, or the patient responds favourably, Phase I therapy should be continued, or the dose of the therapy should be reduced.

If Phase I therapy failed, but the patient falls into good prognostic group, other type of csDMARD; i.e.: leflunomide, or sulfasalazine should be given. If therapy is unsuccessful, biological (b)DMARD (biological originator (bo), or biosimilar (bs), respectively) (anti-TNF, anti-I L6, rituximab) or targeted synthetic (ts) DMARD (e.g. JAK inhibitors) should be given. On the other hand, when Phase I therapy failed and the patient has poor prognostic factors, bDMARD or tsDMARD should be given. When Phase II therapy is failed, another type of bDMARD should be given until complete remission is reached.

Various markers have been proposed to characterize the patient response to treatment with bDMARD therapy. Bystrom J et al. have made cytokine profiling of immune cells and found cells from most anti-TNF responder patients in the current cohort produced higher levels of GM-CSF and TNF pre-treatment than non-responder patients. The authors have suggested that that the disease in responder and non-responder RA patients is likely to be driven/sustained by different inflammatory pathways (Bystrom, Clanchy et al. 2017).

Multidrua _transporters in health and disease

Multidrug resistance (MDR-ABC) transporters (MDRl/P-gp/ABCBl; MRP1/ABCC1; BCRP/ABCG2) are important components in the development of drug resistance in malignancies (Gottesman, Fojo et al. 2002) and in autoimmune conditions, such as RA (Marki-Zay, Tauberne Jakab et al. 2013). Flowever, MDR-ABC transporters also transport a variety of endogenic molecules, such as cytokines and chemokines that play important role in the pathogenesis of RA via influencing cell migration, proliferation and inflammation. Therefore, MDR-ABC transporters may also be important biomarkers of disease progression in RA. The assessment of MDR protein activity may help physicians to evaluate how patients will respond to biological treatment and may support the decision whether there is a necessity to modify the treatment.

The most important csDMARDs; including methotrexate (MTX), sulfasalazine, leflunomide and hydroxychloroquine are substrates of MDR proteins. For this reason, MDR activity of RA patients on csDMARD therapy has been extensively studied and the expression, polymorphisms and activity of drug efflux proteins have been linked to therapeutic success of csDMARDs, especially that of MTX. Tsujimura et al. having analyzed MDR1 expression in lymphocytes of patients with RA who had a long history of sDMARD treatment, found an increase in the levels of MDR1 and correlated MDR1 expression with disease activity and steroid treatment [21]. In 2015, Tsujimura and his colleagues also demonstrated that the expression of MDR1 robustly upregulated on the surface of $CD4^+$ and $CD19^+$ lymphocytes on RA patients as compared with age and gender matched healthy individuals. Furthermore, the expression level of MDR1 was significantly elevated in MTX non-responder patients as compared with responder counterparts. The authors also suggest that treatment by TNF-alpha antagonists probably suppresses transcriptional activation of MDR-1 expression on lymphocytes, and thus inhibition of lymphocyte

activation by TNF antagonists "can probably thwart P-gp-mediated treatment resistance in refractory patients with RA" (Tsujimura and Tanaka 2015).

However, little is known about the relation of MDR proteins to therapeutic success of biological (b)DMARDs, such as anti-TNF agents. The literature is divided whether an association can be found among high MDR1 expression and unresponsiveness to MTX therapy and in general the complex inter-relationship among drug resistance, MDR1 and autoimmunity still remains elusive (Picchianti-Diamanti, Rosado et al. 2014).

(b)DMARDs do not enter the cell, and are therefore not substrates of MDR proteins. While endobiotics, such as the cytokines they target are known to interact with these transporters, such mechanisms are far from being understood (Ronaldson, Ashraf et al. 2010; Garcia-Carrasco, Mendoza-Pinto et al. 2015; Ghandadi and Sahebkar 2016).

Based on current ACR and EULAR guidelines, csDMARD therapy is a first line treatment option for RA patients, in case of non-responsiveness, bDMARD is the second line treatment option (Smolen, Landewe et al. 2017). Thus, it is of particular importance to provide an estimate on patient responsiveness at an early phase or before bDMARD treatment.

A few examples among many efforts to find an appropriate predictor for responsiveness of an anti-TNF-therapy include determining expression level of phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta (PIK3CD) in sample (US20170335367A1), determining the level of Rheumatoid Factor (RF) and/or anti-cyclic citrullinated peptide autoantibody (ACPA) (US20170328897A1), measuring concentration of marker e.g. glycoprotein 130, a cytokine receptor (US20160377612A1), determining baseline serum level of C-X-C motif chemokine 10 and CXCL13 (W02017181038A1). A different approach measure expression of genes or expression of proteins encoded by the genes is described in W02012061620A1.

The research group of the present inventors have studied expression of multidrug transporter in blood samples of RA patients and suggested that low MXR/BCRP/ABCG2 and MRP1/ABCC1 transport activities expressed in MAF values on $CD3^+$ T-lymphocytes may predict the need to start biological therapy in RA patients whose symptoms do not improve on classical DMARD treatment and that a further decrease of $CD3^+$ MXR/BCRP/ABCG2 and increase in $CD3^+$ MRP1/ABCC1 MAF at 12 weeks of bDMARD therapy may indicate a favourable therapeutic response to biological therapy (Szekanecz and Koch 2016).

Multi Drug Resistance (MDR) Protein Activity of T Lymphocytes Assessed by Flow Cytometry is a Predictor of Biological Treatment Response in Rheumatoid Arthritis

A thorough recent review on potential predictors of response to targeted treatment in rheumatoid arthritis concludes that at present, "no biomarkers are known that can predict response to any biologic DMARD in an individual patient with a high level of certainty" (Wijbrandts and Tak 2017).

A further, still later review also carefully collects results on bDMARD response biomarker research in RA and, while admits that available data may guide treatment decisions to a degree, there are limitations and the authors appear to see that in the future high-throughput omics techniques would be more promising (Romao, Vital et al. 2017).

However, in the prior art no proposal has been made whether measurement of MDR1 and/or MRP1 transport activities in the early phase of or before a bDMARD treatment may be appropriate to provide a prediction on the effectiveness or success of bDMARD therapy once csDMARD therapy has failed. In general, further predictors of bDMARD treatment are needed in the art.

BRIEF DESCRIPTION OF THE INVENTION

In an aspect the invention relates to an *in vitro* diagnostic method.

The invention relates to an *in vitro* diagnostic method for assessing the responsiveness of a sDMARD (preferably csDMARD) treated RA patient to bDMARD therapy, said method comprising the steps of

- providing a biological sample of said sDMARD (preferably csDMARD) treated RA patient, said sample comprising $CD3^+$ T-lymphocytes from said patient,
- obtaining one or more transporter activity value(s) by measuring or quantifying transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient, before or at an initial phase of a bDMARD therapy, by using one or more substrate(s) of MDR1, MRP1 or both MDR1 and MRP1,
- comparing the one or more transporter activity value(s) with one or more pre-determined threshold transporter activity level(s),

wherein each pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters and which has been determined using the same one or more substrates,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of each transporter activity value is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of each transporter activity value is not higher than said threshold level.

Preferably said threshold transporter activity level is or has been determined by using the one or more substrates by

measuring or quantifying transport activity of said one or more multidrug transporters in the $CD3^+$ T-lymphocytes in a (reference) patient group known to be responder to the bDMARD therapy and a (reference) patient group known to be non-responder to the bDMARD therapy, and

the transport activity values measured in the responder and non- responder patient groups are analysed (preferably statistically analysed as distributions) to find a threshold level which differentiates between responder transport activity values and non-responder transport activity values.

Preferably the one or more substrates is a substrate of both MDR1 and MRP1 and if transport activity of any of MDR1 and MRP1 is measured separately the other multidrug transporter is inhibited. In a highly preferred embodiment the substrate is a calcein ester, preferably calcein AM.

In a highly preferred embodiment the activity is quantified as a multidrug activity factor (MAF).

Preferably at least the MDR1 activity is measured. In particular embodiment, at least the MRP1 activity is measured. Preferably at least a composite MDR1-MRP1 activity is measured, preferably with a substrate of both MDR1 and MRP1.

Preferably the patient needs a switch or modification of the sDMARD therapy.

In a preferred alternative variant, the invention relates to an *in vitro* diagnostic method for assessing the responsiveness of a sDMARD (preferably csDMARD) treated RA patient to bDMARD therapy, said method comprising the steps of

- providing a biological sample of said sDMARD (preferably csDMARD) treated RA patient, said sample comprising $CD3^+$ T-lymphocytes from said patient,
- obtaining a transporter activity value by measuring transport activity of one or more multidrug transporters comprising at least MDR1, and optionally also MRP1, in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient before a bDMARD therapy or in an initial phase thereof, by using an MDR1 substrate which is optionally also the substrate of MRP1,
- comparing the transporter activity value with a pre-determined threshold transporter activity level, wherein said pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters, and which has been determined, using the same MDR1 substrate,
- considering said RA patient as a non-responder to the bDMARD therapy when the level of the transporter activity value is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of the transporter activity value is not higher than said threshold level.

Said threshold transporter activity level is or has been determined by using the one or more substrates by

measuring or quantifying transport activity of MDR1 and optionally MRP1 in the $CD3^+$ T-lymphocytes in a reference patient group known to be responder to the bDMARD therapy and a reference patient group known to be non-responder to the bDMARD therapy, and

the transport activity values measured in the responder and non-responder patient groups are analysed, preferably statistically analysed as distributions, to find a threshold level which differentiates between responder transport activity values and non-responder transport activity values.

Preferably the MDR1 transporter substrate is also a substrate for MRP1. In this embodiment if MDR1 transporter activity is measured or quantified separately MRP1 is inhibited. If the transporter activity comprises activities of both MDR1 and MRP1, the two transporters are not differentiated by inhibition and a composite activity value is obtained.

In a highly preferred embodiment the substrate is a calcein ester, preferably calcein AM.

In a highly preferred embodiment the activity is quantified as a multidrug activity factor (MAF).

Preferably the transport activity of one or more multidrug transporters is a transport activity of MDR1.

Preferably the transport activity of MDR1 is measured by

- an MDR1 substrate is specific to MDR1 or
- an MDR1 substrate which is the substrate of at least one other multidrug transporter which is inhibited in the measurement by an inhibitor of said at least one other multidrug transporter (e.g. with an inhibitor which does not inhibit MDR1 or with an inhibitor specific to the least one other multidrug transporter).

In a preferred embodiment the transport activity of one or more multidrug transporters is a composite transport activity of MDR1 and MRP1. Preferably the transport activity of MDR1 is measured by an MDR1 substrate which is also the substrate of MRP1.

In a preferred alternative variant, the invention relates to an *in vitro* diagnostic method of the invention for assessing the responsiveness of a sDMARD (preferably csDMARD) treated RA patient to bDMARD (bo or bs) therapy, wherein the sDMARD treated RA patient is in need of a switch or modification of the therapy, said method comprising the steps of

- providing a biological sample of said sDMARD (preferably csDMARD) treated RA patient, said sample comprising lymphocytes from said patient, said lymphocytes including at least $CD3^+$ T-lymphocytes, i.e. said sample is a lymphocyte containing biological sample,
- measuring, as a transporter activity, at least a composite transporter activity of MDR1 and MRP1 transporter proteins (composite transporter activity, preferably MAF_C) in $CD3^+$ T-lymphocytes of said RA patient before starting bDMARD therapy, or at an initial phase of said bDMARD therapy at the latest, thereby obtaining a value for the composite MDR1 MRP1 transporter activity (composite MDR1 MRP1 transporter activity value),
- comparing the composite MDR1 MRP1 transporter activity value (preferably (MAF_C) with a pre-determined threshold transporter activity level,

wherein said pre-determined threshold transporter activity level is a threshold value for the composite MDR1 MRP1 transporter activity, and which has been determined by a test in which the (reference) patient group known to be responder to the bDMARD therapy and the (reference) patient group non-responder to the bDMARD therapy have composite MDR1 and MRP1 transporter activities with different statistical distributions, and thus can be or are differentiated based on their composite MDR1 and MRP1 transporter activity,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of the composite transporter activity is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of the composite transporter activity is not higher than said threshold level.

In the method of the invention preferably the sDMARD treated RA patient needs a switch or modification of the therapy.

In the method of the invention preferably said biological sample is a leukocyte containing biological sample wherein the leukocytes comprise lymphocytes, preferably a blood sample. Said lymphocytes include $CD3^+$ T-lymphocytes.

In the method of the invention preferably measuring in particular, comprises or includes quantifying of transport activity.

In a preferred embodiment MDR1 activity, MRP1 activity and composite MDR1 and MRP1 activity are each measured and the patients are considered as non-responders if each of the transporter activity values are above threshold, and responders if each of the transporter activity values are not higher than the above threshold.

In the method of the invention preferably the initial phase of the bDMARD therapy means that the method is carried out in the first 8 weeks, preferably in the first 7 or 6 weeks, or preferably the first 4 weeks or highly preferably in the first two weeks of or in the first week of the bDMARD therapy.

In particular, in an alternative wording, the *in vitro* (diagnostic) method is for determining predisposition of a sDMARD treated RA patient to respond (or not) to bDMARD therapy. In particular, the in vitro (diagnostic) method is for predicting the expectable success or effectiveness of a sDMARD treated RA patient to respond to bDMARD therapy.

In particular, a transporter activity value is obtained by measuring transport activity of MDR1 or MRP1 or a composite of the transport activities of MDR1 and MRP1.

In a highly preferred embodiment the method of any of claim 1 wherein the threshold value is obtained by an ROC analysis.

In a particular embodiment the responder and non-responder patient groups are established by a DAS28 score value.

In a preferred embodiment in the *in vitro* diagnostic method of the invention the bDMARD therapy is selected from the group consisting of

- anti-TNF therapy (in particular, a monoclonal antibody against TNF),
- T-cell activation inhibitor therapy, e.g. by a costimulation inhibitor, preferably a protein, e.g. fusion protein, binding to CD80 and CD86 molecules (in particular, abatacept),
- anti-B-lymphocyte proliferation therapy (in particular, rituximab),
- anti-IL6 therapy, preferably an IL-6 receptor blocker (in particular tocilizumab, sarilumab) or preferably an IL-6 inhibitor (in particular clazakizumab, sirukumab).

Preferably the bDMARD therapy is anti-TNF therapy or a T-cell activation inhibitor therapy, highly preferably the bDMARD therapy is anti-TNF therapy.

Preferably the anti-TNF therapy may comprise the administration of any of the following drugs: adalimumab, certolizumab pegol, etanercept, golimumab, infliximab as boDMARD and their biosimilars.

In a particular preferred embodiment of the method of the invention if the patient is assessed to be non-responsive to an anti-TNF therapy or a T-cell activation inhibitor therapy (preferably an anti-TNF therapy), then a different bDMARD therapy selected from

- anti-B-lymphocyte proliferation therapy (for example rituximab),
- anti-IL6 therapy (for example tocilizumab)

which is considered as a modified therapy.

In a particular preferred embodiment, if the patient is assessed to be non-responsive to a bDMARD therapy, including an anti-TNF therapy or a T-cell activation inhibitor therapy, then a different tsDMARD is considered as a modified therapy.

In a preferred embodiment of the method of the invention measuring the transporter activity comprises

- contacting at least the $CD3^+$ T-lymphocytes in the biological sample with the one or more transporter substrate(s), said substrate being a derivative of a detectable fluorescent compound, and wherein said derivative is taken up by at least the $CD3^+$ T-lymphocytes and is hydrolyzed into said fluorescent compound in the cells, wherein said fluorescent compound is not transportable by MDR1 or MRP1 or by neither MDR1 nor MRP1 or transportable to a significantly lesser extent than the derivative, preferably the ester derivative, and
- measuring fluorescence in the $CD3^+$ T-lymphocytes, preferably after labelling them with fluorochrome-conjugated anti-CD3 antibodies,
- obtaining or calculating the transport activity value from the fluorescence in the $CD3^+$ T-lymphocytes.

Preferably measurement is carried out by flow cytometry.

In a preferred embodiment at least MDR1 activity is measured.

In a preferred embodiment, a composite transporter activity is measured, preferably a composite MDR1 + MRP1 activity.

In an embodiment, the $CD3^+$ T lymphocytes are collected before transporter activity is measured and thus transporter activity is measured selectively in the $CD3^+$ T lymphocytes.

In another embodiment, the transporter substrate is added to the cells before collecting the $CD3^+$ T lymphocytes (from the biological sample, preferably blood sample) and said $CD3^+$ T lymphocytes are labelled with fluorescently labelled antibodies and fluorescence of the substrate is thus measured in the $CD3^+$ T-lymphocytes.

In appropriate embodiments said derivative is a transportable substrate for both MDR1 and MRP1.

Preferably the MDR1 transporter substrate is added to the biological sample.

In a preferred embodiment the measuring or quantifying the transport activity comprises

- contacting at least the $CD3^+$ T-lymphocytes in the biological sample with an ester derivative of a detectable fluorescent compound, preferably a calcein ester, wherein said derivative is taken up by at least the $CD3^+$ T-lymphocytes and is hydrolyzed into said fluorescent compound in the cells, wherein said fluorescent compound is neither transportable by MDR1 nor MRP1 or transportable to a significantly lesser extent than the ester derivative, and
- measuring fluorescence in the $CD3^+$ T-lymphocytes, preferably after labelling them with fluorochrome-conjugated anti-CD3 antibodies,
- obtaining the composite transporter activity (preferably MAF_C) value from the fluorescence in the $CD3^+$ T-lymphocytes.

In a highly preferred embodiment the substrate, preferably the detectable fluorescent ester compound is calcein and/or measuring transport activity is based on the difference between the fluorescence of

the detectable fluorescent compound in the cells measured in the presence of an inhibitor, preferably a selective inhibitor, of the multidrug transporters and the fluorescence measured in absence of said inhibitor. Preferably the measurement is carried out by flow cytometry.

Preferably, the fluorescence of the detectable fluorescent compound in the cells measured in the presence of an inhibitor of MDR1 and MRP1 of the multidrug transporters and/or the fluorescence is measured in the presence of a specific MRP1 inhibitor wherein MDR1 is not inhibited. Preferably the MDR1 transporter activity (value) is calculated as an MDR activity factor ($MAF_{MDR}I$) and preferably the MRP1 transporter activity (value) is calculated as an MRP1 activity factor ($MAF_{MRP}I$).

Preferably, the fluorescence of the detectable fluorescent compound in the cells measured in the presence of an inhibitor of MDR1 and MRP1 of the multidrug transporters and the fluorescence measured in absence of said inhibitor; wherein preferably the (quantitative) composite MDR1 MRP1 transporter activity (value) is a composite MDR activity factor (MAF_C).

In a preferred embodiment of the methods wherein transporter activity is quantified as a MAF value the biological sample is blood and the MDR1 substrate is calcein and the MDR1 transporter activity (value) threshold level is calculated as an MDR activity factor for MDR1 ($MAF_{MDR}I$) and said threshold (in MAF percentage) is between 15 to 19, preferably 16 to 19 or 16 to 18, more preferably between 17 to 19 or 17 to 18. In a further preferred embodiment the biological sample is blood and the MDR1 substrate is calcein, and the MDR1 transporter activity (value) threshold level is calculated as an MDR activity factor (MAF_{MDR}) and said RA patient is considered as a non-responder to biological therapy wherein the level of MAF_{MDR} is above a MAF_{MDR} threshold from 15 to 19, preferably 16 to 19 or 16 to 18, more preferably between 17 to 19 or 17 to 18.

In a preferred embodiment of the methods wherein the biological sample is blood and the MDR1 substrate is calcein and the MDR1 transporter activity (value) threshold level is calculated as a composite. The cumulative MDR activity factor for MDR1 and MRP1 (MAF_c) and said threshold is between 19 to 23, preferably 20 to 22 more preferably 21.3. In a further preferred embodiment the biological sample is blood and MDR1 substrate is calcein and the MDR1 transporter activity (value) threshold level is calculated as a composite MDR activity factor for MDR1 and MRP1 (MAF_c) and said RA patient is considered as a non-responder to biological therapy wherein the level of MAFc is above a MAFc threshold from 19 to 23, preferably 21.3.

In a further variant of the method a further measurement of a transporter activity as defined above is also performed. Such measurement may be performed after the above defined one or more measurement. Such measurement may provide additional information about the responsiveness of the patient for the bDMARD therapy.

In an embodiment said sDMARD-treated RA patient has been also treated by bDMARD therapy, said method additionally comprising

- providing a further biological sample of said sDMARD-treated RA patient between weeks 4 and 7 of the bDMARD therapy,

- obtaining one or more further transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient between weeks 4 and 7 of the bDMARD therapy, by using one or more substrate(s) of MDR1, MRP1 or both MDR1 and MRP1,
- comparing one or more further transporter activity value(s) with one or more pre-determined threshold transporter activity level(s),

wherein each pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters and which has been determined using the same one or more substrates, as defined above,

- considering said RA patient as a non-responder to the biological therapy wherein the level of each transporter activity value is above said each threshold level, and
- considering said RA patient as a responder to the biological therapy wherein the level of the MDR1 transporter activity value is not higher than said each threshold level.

Preferably, the bDMARD therapy is a bDMARD therapy as defined above or herein.

Preferably measuring/quantifying transporter activity of transporter protein is carried out as defined in any of the paragraphs as defined above for the transport activity measurement.

In a highly preferred embodiment

- the detectable fluorescent ester compound is calcein ester and/or
- the (quantitative) composite transporter activity value is based on the difference between the fluorescence of the detectable fluorescent compound in the cells measured in the presence of an inhibitor of the multidrug transporters and the fluorescence measured in absence of said inhibitor.

Preferably the (quantitative) transporter activity (value) is MAF value.

In a highly preferred embodiment in measurement or quantifying step, said RA patient is considered as a non-responder to biological therapy wherein upon a measurement between 4 to 7 weeks preferably at about 6 weeks of the bDMARD therapy

- at least the MDR1 activity is measured, and the transporter activity value is MAF_{mdr1} value, and

said RA patient is considered as a non-responder to biological therapy wherein the level of MAF_{MDR1}.is above a MAF_{mdr1} threshold from 12 to 15, preferably from 13 to 14, more preferably above a threshold of about 13.9;

- at least the MRP1 activity is measured, and the transporter activity value is MAF_{mrp1} value, and

said RA patient is considered as a non-responder to biological therapy wherein the level of MAF_{MDR1}.is above a MAF_{mdr1} threshold from 5 to 7, preferably from 5.5 to 6.5, more preferably above a threshold of about 6.0.

- at least a composite activity is measured, and the transporter activity value is MAF_C value, and

said RA patient is considered as a non-responder to biological therapy wherein the level of MAF_c.is above a MAF_{mdr1} threshold from 18 to 22, preferably from 19 to 21, more preferably above a threshold of about 20.3.

In a further embodiment the threshold values are set above any of the value as defined above thereby increasing the Preferably the biological sample is blood sample.

Preferably the patient is a mammal, preferably a human.

In a preferred embodiment MDR1 activity, MRP1 activity and composite MDR1 and MRP1 activity are each measured in this stage and the patients are considered as non-responders if each of the transporter activity values are above threshold, and responders if each of the transporter activity values are not higher than the above threshold.

In a further aspect the invention relates to a method for therapy including assessing the responsiveness of a sDMARD (preferably csDMARD) treated RA patient to bDMARD therapy said method comprising the steps of

- providing a biological sample of said sDMARD (preferably csDMARD) treated RA patient, said sample comprising $CD3^+$ T-lymphocytes from said patient,
- obtaining one or more transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient, before or at an initial phase of a bDMARD therapy, by using one or more substrate(s) of MDR1, MRP1 or both MDR1 and MRP1,
- comparing the one or more transporter activity value(s) with one or more pre-determined threshold transporter activity level(s),

wherein each pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters and which has been determined using the same one or more substrates,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of each transporter activity value is above said threshold level and applying alternative therapy, in particular, an alternative csDMARS therapy or, preferably a tsDMARD therapy;
- considering said RA patient as a responder to the bDMARD therapy when the level of each transporter activity value is not higher than said threshold level and applying a bDMARD therapy.

Preferably the therapy comprises any of the diagnostic methods for assessing the responsiveness of a sDMARD (preferably csDMARD) treated RA patient to bDMARD therapy as defined above.

Preferably the bDMARD therapy is as defined above.

In particular, the bDMARD therapy is selected from the group consisting of

- anti-TNF therapy (in particular, a monoclonal antibody against TNF),
- T-cell activation inhibitor therapy, preferably a protein, e.g. fusion protein, binding to CD80 and CD86 molecules (in particular abatacept),
- costimulation inhibitor (in particular abatacept);
- anti-B-lymphocyte proliferation therapy (in particular rituximab),
- anti-l L6 therapy, preferably an IL-6 receptor blocker (in particular tocilizumab, sarilumab) or preferably an IL-6 inhibitor (in particular clazakizumab, sirukumab).

Preferably the bDMARD therapy is anti-TNF therapy or a T-cell activation inhibitor therapy, highly preferably the bDMARD therapy is anti-TNF therapy.

Preferably the anti-TNF therapy may comprise the administration of any of the following drugs: adalimumab, certolizumab pegol, etanercept, golimumab, infliximab as boDMARD and their biosimilars.

In a particular, preferred embodiment if the patient is assessed to be non-responsive to an anti-TNF therapy or a T-cell activation inhibitor therapy, preferably an anti-TNF therapy, then a different bDMARD therapy selected from

- anti-B-lymphocyte proliferation therapy,
- anti-IL6 therapy (for example tocilizumab),

which is considered as a modified therapy.

In a particular preferred embodiment, if the patient is assessed to be non-responsive to a bDMARD therapy, including an anti-TNF therapy or a T-cell activation inhibitor therapy, then a different tsDMARD is considered as a modified therapy.

Preferably the bDMARD therapy is anti-TNF therapy.

Preferably the measuring or quantifying the multidrug transporter activity, in particular, the MDR1 activity, MRP1 activity and/or the composite MDR1 MRP1 transporter activity is carried out as defined above.

In a further aspect the invention relates to a use of a kit for assessing the responsiveness of a sDMARD (preferably csDMARD) treated RA patient to bDMARD therapy before or at an initial phase of the bDMARD therapy, or for a purpose as defined herein,

by obtaining one or more transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient,

wherein

said RA patient is considered as a non-responder to the bDMARD therapy when the level of each transporter activity value is above a respective threshold level, or for a purposed, and

considering said RA patient as a responder to the bDMARD therapy when the level of each transporter activity value is not higher than a respective threshold level,

said kit comprising

- one or more substrate(s) of MDR1, MRP1 or both MDR1 and MRP1 for the measuring of the respective transporter activity

said substrate being taken up by $CD3^+$ T-lymphocytes once contacted with them in a biological sample, wherein preferably said substrate is detectable, preferably fluorescent,

- label for $CD3^+$ T-lymphocytes,

and preferably

- inhibitor for MRP1 and/or,
- inhibitor for MDR1.

Preferably the kit as defined above also comprises instructions to carry out the method of the invention.

In a preferred embodiment said use of the kit is for assessing the responsiveness of a sDMARD (preferably csDMARD) treated RA patient to bDMARD therapy before or at an initial phase of the bDMARD therapy as defined above,

for use in a method as defined herein or above or in a method according to of any of the in vitro diagnostic methods as defined above, wherein said kit comprises,

- a substrate for MDR1 and MRP1 for the measuring of a composite MDR1 and MRP1 transport activity, said substrate being taken up by leukocytes (preferably $CD3^+$ T-lymphocytes once contacted), in a biological sample,

wherein preferably said substrate is detectable, preferably fluorescent,

- label for $CD3^+$ T-lymphocytes, preferably a $CD3^+$ T-lymphocyte specific antibody,

and preferably

- inhibitor for MRP1,

- inhibitor for one or more other multidrug transporter.

In an embodiment the kit also comprises an inhibitor for BCRP.

Preferably said threshold transporter activity level is or has been determined by using the one or more substrates by

measuring/quantifying transport activity of said one or more multidrug transporters in the $CD3^+$ T-lymphocytes in a (reference) patient group known to be responder to the bDMARD therapy and a (reference) patient group known to be non-responder to the bDMARD therapy, and

the transport activity values measured in the responder and non- responder patient groups are analysed (preferably statistically analysed as distributions) to find a threshold level which differentiates between responder transport activity values and non-responder transport activity values.

Preferably the one or more substrates is a substrate of both MDR1 and MRP1 and if transport activity of any of MDR1 and MRP1 is measured separately the other multidrug transporter is inhibited. In a highly preferred embodiment the substrate is a calcein ester, preferably calcein AM.

In a highly preferred embodiment the activity is quantified as a multidrug activity factor (MAF).

Preferably at least the MDR1 activity is measured. In a particular embodiment at least the MRP1 activity is measured. Preferably at least a composite MDR1-MRP1 activity is measured, preferably with a substrate of both MDR1 and MRP1.

Preferably the patient needs switch or modification of the sDMARD therapy.

In a preferred embodiment a transporter activity value is obtained by measuring transport activity of one or more multidrug transporters comprising at least MDR1, and optionally also MRP1, in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient before a bDMARD therapy or in an initial phase thereof, by using an MDR1 substrate which is optionally also the substrate of MRP1,

Preferably the MDR1 transporter substrate is also a substrate for MRP1. In this embodiment if MDR1 transporter activity is measured or quantified separately MRP1 is inhibited. If the transporter activity

comprises activities of both MDR1 and MRP1, the two transporters are not differentiated by inhibition and a composite activity value is obtained.

In a highly preferred embodiment the substrate is a calcein ester, preferably calcein AM.

In a highly preferred embodiment the activity is quantified as a multridrug activity factor (MAF).

In the present invention preferably a bDMARD therapy involves both boDMARD and bsDMARD therapy.

In a highly preferred embodiment the kit measures the drug transport activity of at least two subfamilies of multidrug resistance proteins: MDR1 and MRP1,

utilizes calcein-AM, a substrate for targeted extrusion by multi-drug transporters, and

the degree of fluorescence is observed.

In an embodiment the kit also comprises MDR1 and/or MRP1 inhibitors, preferably selected from the group consisting of, verapamil, oligomycin, or cyclosporine, preferably verapamil and indomethacin are present.

DEFINITIONS

A "detectable fluorescent compound" as used herein is a compound which can be detected by irradiating with an UV or VIS electromagnetic radiation ("irradiating light") and the compound absorbs the irradiating light and emits light (emitted light) at another, preferably longer wavelength than that of the irradiating light. Preferably the "detectable fluorescent compound" is capable of fluorescence i.e. emission of light inside of i.e. within a cell.

The "derivative of a detectable fluorescent compound" relates to a chemical compound that is derived from said detectable fluorescent compound by an actual (not only theoretical) chemical reaction, preferably an actually performed chemical reaction, and from which the original detectable non-fluorescent compound can be regained by a chemical reaction. Preferably the derivative is fluorescent. Preferably derivative has one or more, preferably more, preferably all of the following features: the derivative is substrate to a MDR protein, is hydrophobic, is permeable to cell membranes and can enter a cell by diffusion through the membrane.

"Ester derivative" of a detectable fluorescent compound relates to a derivative wherein upon the chemical reaction an ester is formed wherein preferably the detectable fluorescent compound can be formed again by ester hydrolyses, preferably by intracellular esterases. Preferably the ester derivative is fluorescent. Preferably the ester derivative is substrate to a MDR protein.

Preferably inside the cell the derivative compound is cleaved by intracellular enzymes, in case of ester derivatives by esterase activity, resulting in a fluorescent non-membrane permeable form of the derivative, which is preferably hydrophilic or charged, and which is preferably not a substrate of the transporter protein.

A "calcein derivative compound", as used herein, refers to a derivative of calcein (CAS No. 1461-15-0, alternative name: fluorexon) with the properties, e.g., of being a substrate to a MDR protein; being permeable to cell membranes, so as to diffuse through the extracellular membrane and enter a cell; and having low sensitivity to Ca^{2+} ions, Mg^{2+} ions, and pH.

For example, in case of the non-fluorescent acetomethoxy derivate of calcein (calcein AM, AM = acetoxymethyl) after it is passively diffused into the cells, intracellular esterases remove the acetomethoxy group, the molecule gets trapped inside as calcein is not a substrate of MDR1 and MRP1, and gives out strong green fluorescence.

Calcein derivatives compounds include, but are not limited to, acetoxymethyl esters of calcein, e.g., calcein-AM, calcein blue AM, or carboxycalcein blue AM, or an acetate ester of calcein [see, e.g., Haugland, Richard P. Handbook of fluorescent probes and research products. Molecular Probes, Inc; 9th edition (2002)].

"Measuring" or "measurement" is understood herein as quantitative characterization of a physical object or entity or a multitude (population or plurality) thereof, or their function or quantitative characterization of a physical or chemical process, comprising the assignment of a quantity, value, e.g. a numerical value or a number characteristic of the object or entity or multitude or function or process, by comparison with units and, in comparison with other object or entity or multitude or function or process. Preferably a measurement is consistent with methods known in the art or the international guidelines of metrology. The magnitude is the numerical value of the characterization, usually obtained with a suitably chosen measuring instrument, whereas the unit assigns a mathematical weighting factor to the magnitude that is derived as a ratio to the property of an artefact used as standard unit or a natural physical quantity as unit.

"Quantifying" or "quantification" or "quantitation" is understood herein as an assignment of a physical quantity to a physical object or entity or a multitude (population or plurality) thereof, or their function or quantitative characterization of a physical or chemical process, expressed in a numerical value or number and units, and, in comparison with other object or entity. Preferably "quantifying" or "quantification" is a measurement or an essential part of a measurement.

The measurement has an uncertainty which may represent the random and systemic errors of the measurement procedure. The skilled person is aware of this and can handle this error in view of the measurement or quantification applied.

"Comparing" two levels preferably two activity levels are understood herein to include a comparison of quantities expressed in numerical values characterizing said levels to establish which is higher or lower, or establishing a difference or establishing a ratio of the levels, or values derived from the levels, optionally completed with other mathematical procedures as the quantification or calculation method requires.

A "membrane transporter" is a membrane integrated protein, which is permanently anchored in the membrane having a membrane spanning part and having parts on both sides of the membrane, wherein it is capable of transporting, e.g. exporting or extruding or importing entities, either actively or passively through the membrane into which it is integrated in. The entity can be e.g. a molecule or a molecule ion which is preferably fluorescent.

"ABC transporter" stands for ATP-binding cassette transporters which are a superfamily of membrane transporters that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain

biological processes including transport of entities across membranes. Denominations and subfamilies of ABC transporters are used herein as assigned by the HUGO Gene Nomenclature Committee (HGNC). For example, membrane transporters of the "ABCG family" belong to the G subfamily of ABC transporters consisting of half-transporters, which oligomerise to form the functional transporter.

A "multidrug transporter" is an ABC transporter, also mentioned herein as an ABC multidrug transporter, which can transport from the cell, in the membrane of which it is present, a multiplicity or preferably a wide variety of chemical compounds.

"Multidrug resistance", as used herein, refers to the ability of cells to develop resistance to a broad range of structurally or functionally unrelated drugs by multidrug transporter(s). Preferably, "multi-drug resistance" refers to the state which is dependent on expression or overexpression of MDR1, MRP1, or a related homologue, and/or on amplification of a gene encoding said multi-drug transporter protein.

"ABC transporter activity", i.e. the "activity" of an ABC transporter protein refers to any activity exerted by the said transporter protein including e.g. its biological function, "transport activity", i.e. transport of a drug through the membrane carrying the said protein, or ATP-ase activity, as far as it is an indicator of transport activity, like substrate stimulated ATP-ase activity. Preferably the activity measured in the present invention characterized or is related to or correlates to transport activity of the multidrug transporter.

A "substrate" of an ABC transporter protein is a compound that can be transporter from the cell through an ABC transporter mediated active transport mechanism.

In a preferred embodiment the ABC multidrug transporters the activity of which is measured in the present invention are selected from the following transporters:

ABCB1 (MDR1) which belongs to the "ABCB family" belong to the B subfamily of ABC transporters;

ABCC1 (MRP1) which belongs to Multidrug Resistance Proteins (MRPs) of the "ABCC family" of the C subfamily of ABC transporters;

and in a preferred embodiment ABCG2 (other names among others: BRCP, MXR1, CDw338) which belong to membrane transporters of the "ABCG family" i.e. of the G subfamily of ABC transporters consisting of half-transporters, which oligomerise to form the functional transporter.

By "measuring transport activity" of a multidrug transporter it is understood that in cells or in a population of cells in which the given multidrug transporter resides or assumably resides the transport activity is measured and/or quantified wherein preferably the activity is total or overall transport activity of one or more multidrug transporter(s). In a preferred embodiment if the expression of the transporters is increased the activity also increases.

In general, any physical quantity which quantitatively characterizes the transport activity of the multidrug transporter can be applied in the present invention. In a preferred embodiment the physical quantity is obtained by comparison of a value obtained for cells in which the multidrug transporter is active with a value obtained for cells in which it is inhibited.

In a preferred embodiment the activity is measured via or with a substrate compound which is able to get through the cell membrane and to be transported from the cell in which the transporter resides.

In a particular preferred embodiment the activity is measured via or with a substrate compound which is a "derivative of a detectable fluorescent compound" and is able to get through the cell membrane and to be transported from the cell in which the transporter resides, wherein within the cell the derivative is converted to the detectable fluorescent compound which is non-membrane permeable, which is preferably hydrophilic or charged, and which is preferably not a substrate of the transporter protein. Preferably the derivative is an ester derivative. Highly preferably the derivative is a "calcein derivative compound". Preferably the detectable fluorescent compound is detected within the cell.

Upon measuring or quantifying (or any equivalent expression) transport activity in a preferred embodiment the activity is quantitatively characterized by a factor which comprises or is related or is proportional to the difference between the fluorescence of the detectable fluorescent compound in the cells measured in the presence of an inhibitor of the one or more membrane transporter and the fluorescence measured in absence of said inhibitor i.e. when the transporter is active. This difference is higher if the transporter is active because the fluorescence provide by the compound in the cell in lack of the inhibitor is lower as the compound (or in case its derivative is applied the derivative) is transported.

By "measuring calcein compound" is meant determining the amount of the calcein compound which accumulates in a cell as an inverse indication of the amount of calcein derivative extruded from the cell by a multi-drug transporter protein.

Techniques for measuring intracellular calcein include, but are not limited to, flow cytometry, fluorimetry, or cell imaging. Use of calcein as a fluorescent probe, in combination with these techniques, provides a quantitative, functional assay of activity of certain multidrug transporter, e.g. of MDR1 and MRP1 activity. By "exposing" is meant placing the calcein compound in the environment of the cells of the biological specimen, e.g., by adding the calcein compound to the media in which the cells of the biological specimen are incubated, so as to allow the calcein compound to enter the cells.

In a preferred embodiment the transport activity of the MDR transporter is measured via the transport (extrusion) of the derivative compound as the difference between the amount of the dye accumulated in the presence and absence of inhibitors. The fluorescence measurement in the presence of an inhibitor constitutes the maximal (potential) fluorescence (F_{max}) with the given cell population when the multidrug transporters are rendered non-functional. The fluorescence measurement in the absence of an inhibitor constitutes the minimal fluorescence (F_0) with the given cell population when the multidrug transporters are functional. This represents a standardization method, which eliminates unknown cell type-specific variables that influence cellular calcein accumulation, such as esterase activity, cell size, etc.

In a preferred embodiment quantitation or quantifying of this fluorescence is carried out through the development of the MDR Activity Factor (MAF) which is calculated as the ratio of the said difference ($F_{max} - F_0$) and of the maximal fluorescence, i.e.

$MAF = (F_{max} - F_o) / F_{max}$

or if expressed in percentage, $MAF = 100 \times (F_{max} - F_o) / F_{max}$.

MAF in percentage is often given as MAF%, however, in the present description this is not indicated, however, the MAF values given herein are given in percentage unless otherwise indicated.

The transport activity of MDR1 and MRP1 can be easily distinguished with selective inhibitors. Optionally other membrane transporters can be inhibited.

By "kit" is meant a package, collection, or container of materials intended to aid one in use of the assay of the invention. By "instructions" is meant a list of steps, or a description of the invention, intended to instruct a practitioner, e.g., a laboratory clinician or technician, to conduct an assay of the invention. The instructions can be written, oral (e.g., on an audio tape medium), or visual (e.g., on a video tape medium).

By a "biological sample", is meant a sample comprising living immune cells obtained from a mammal and optionally processed. The biological sample can be isolated from the mammal as a body fluid, preferably blood or synovial fluid. Preferably the biological sample is a blood sample. Preferred immune cells are at least T-lymphocytes and/or T lymphocyte subsets and optionally or additionally B-cells.

A "patient" is a subject, i.e. a is an individual of a human or a mammalian species who is or intended to be under medical or veterinarian observation, supervision, diagnosis or treatment of a condition. Preferably the individual is a primate, a hominid or a human.

A "treatment" refers to any process, action, application, therapy, or the like, wherein the subject or patient is under aid, in particular, medical, or veterinarian aid with the object of improving the subject's or patient's condition, either directly or indirectly.

A "therapy" is understood herein as a method for treatment in which a given medicament or pharmaceutical composition is administered to said patient, preferably administered for a certain period of time with the object of improving the subject's or patient's condition.

A "sDMARD" is a "synthetic disease-modifying antirheumatic drug", which is a synthetic chemical compound defined by their use in rheumatoid arthritis to slow down disease progression by targeting the immune system or any immune system pathway and by a mechanism other than lowering inflammation specifically. csDMARD are different from NSAID (non-steroidal anti -inflammatory drugs).

A "csDMARD" is a "classic synthetic" or "conventional synthetic disease-modifying antirheumatic drug", preferably with a broad spectrum, which is a synthetic chemical compound defined by their use in rheumatoid arthritis to slow down disease progression by targeting the immune system, typically broadly or in a way not specified yet, and which has been developed not specifically to target JAK inhibition or a specific pathway inside immune cells. Thus, csDMARD are a subgroup of sDMARDs (Smolen, Landewe et al. 2017).

In a preferred embodiment the csDMARD is selected from the group of compounds consisting of azathioprine, cyclophosphamide (also used in lupus in patients who do not respond to traditional therapy or who experience kidney damage), cyclosporine (used sometimes for lupus in people who do not respond to other therapies), hydroxychloroquine sulfate (an antimalarial drug), leflunomide (people who cannot tolerate methotrexate may take leflunomide. It can also be taken in combination with methotrexate), methotrexate, mycophenolate mofetil (may be used in people whose RA does not

respond to other therapies), sulfasalazine (may be used in a triple therapy combination for RA (methotrexate, sulfasalazine, hydroxychloroquine), preferably selected from methotrexate, chloroquine and salazopryne and optionally glucocorticoids.

In a preferred embodiment the definition of "csDMARD" does not involve glucocorticoids.

A "tsDMARD" is a "targeted synthetic disease-modifying antirheumatic drug", which is a synthetic chemical compound which has been developed to specifically target the JAK kinase pathway

In a preferred embodiment the tsDMARD is selected from the group consisting of baricitinib, apremilast (Otezla) and tofacitinib (Xeljanz).

A "bDMARD" is a "biological synthetic disease-modifying antirheumatic drug", which is a biological molecule produced by living cells and which has been developed to block an important mediator participating in the development or in the maintenance of chronic inflammation. The term bDMARDs involve biological originator (bo) and biosimilar (bs) DMARDs. A boDMARD and its corresponding bsDMARD are, nevertheless, expectably equivalent from the point of view of the present invention. Based on their targets, bDMARDS include:

- tumour necrosis factor (TNF)-inhibitors (adalimumab, certolizumab pegol, etanercept, golimumab, infliximab as boDMARD and their biosimilars),
- costimulation inhibitor (abatacept);
- IL-6 receptor blocker (tocilizumab, sarilumab)
- IL-6 inhibitors (clazakizumab, sirukumab)
- anti-B cell agent (rituximab)

-"Switch" of a therapy means an alteration of the therapy which comprises the application of a medicament or pharmaceutical composition which has not been applied previously. In an embodiment it involves the parallel abandonment of the medicament or pharmaceutical composition administered previously to said patient. In another embodiment it involves the continued administration of the medicament or pharmaceutical composition administered previously to said patient, either in a modified or in an unmodified doses or regime.

Preferably the condition is rheumatoid arthritis.

In particular, a "switch of the therapy" or an "alteration of the therapy", as used herein, relates to an initial DMARD therapy of rheumatoid arthritis, and comprises

- a modification of sDMARD therapy either to a combination of the same sDMARD (or a combination of sDMARDs) and a bDMARD, or to a combination of a different sDMARD or a different combination of sDMARDs and a bDMARD;
- a modification of sDMARD therapy to a therapy with a bDMARD alone, or
- another sDMARD, in particular a tsDMARD;

wherein preferably the initial DMARD therapy is an csDMARD therapy.

Highly preferably the initial csDMARD therapy is methotrexate therapy.

"Assessing" the success or outcome of a treatment or therapy is understood herein as a method, e.g. a diagnostic type method resulting in a quantitative value which predicts whether a given treatment or

therapy will be effective to a given patient and thereby may contribute to a decision on the way of treatment or therapy in the future or on the continuation or alteration of it. Assessing normally involves measurement including calculation and preferably involves consideration of the results and/or drawing conclusion.

As used herein the singular forms "a", "an" and if context allows "the" include plural forms as well unless the context dictates otherwise.

The term "comprises" or "comprising" or "including" are to be construed here as having a non-exhaustive meaning and allow the addition or involvement of further features or method steps or components to anything which comprises the listed features or method steps or components.

The expression "consisting essentially of" or "comprising substantially" is to be understood as consisting of mandatory features or method steps or components listed in a list e.g. in a claim whereas allowing to contain additionally other features or method steps or components which do not materially affect the essential characteristics of the use, method, composition or other subject matter. It is to be understood that "comprises" or "comprising" or "including" can be replaced herein by "consisting essentially of" or "comprising substantially" if so required without addition of new matter.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. DAS28 values at different sampling time points in Responder (A) and Non-responder (B) RA patients. *$p < 0.05$ vs. 0 wk. DAS28 is a scoring system to determine activity of RA based on clinical symptoms and quality of life of patients. While an improvement can be observed with decreasing scores in Responders during bDMARD treatment, this improvement is not present in Non-responders.

Figure 2. Activity of the investigated transporters on $CD3^+$ cells in controls as well as before (A.I-4) and at 6 weeks after (B.I-4) the start of bDMARD therapy in RA patients. *$p < 0.05$ vs. Responder.

Figure 3. ROC analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6 wk. Patients with MAF values above the respective cut-off thresholds are likely to be Non-responders to treatment. (A) MAF_C of $CD3^+$ cells at 6 wk: $p = 0.033$, AUC = 0.72; (B) MAF_C of $CD3^+$ cells at 0 wk: $p = 0.043$, AUC = 0.68; (C) MAF_{MRPI} on $CD3^+$ cells at 6 wk: $p = 0.049$, AUC = 0.69; (D) MAF_{MDRI} on $CD3^+$ cells at 6wk: $p = 0.048$, AUC = 0.70.

Figure 4. ROC analysis was also performed to determine the predictive value of MAF_{mdr1} at the time of diagnosis (0 week); in comparison with MAF_C of $CD3^+$ cells at 0 wk (A). When MAF_{mdr1} of $CD3^+$ T lymphocytes is above 17.4 (B), RA patients are likely to be non-responder to bDMARD treatment. Although statistical significance is not present ($p=0.24$) the sensitivity (58.3%) and the specificity (81.5%) are high enough to use this value as a treatment prediction marker.

The curves demonstrate cut-off values based on various sensitivity and specificity values. The closer we are to the upper left corner of the graph, the more specific and sensitive the cut-off value is. Since no test with perfect specificity and sensitivity exists in real life, a compromise needs to be made against variable specificity and sensitivity values. In our calculations, these values were chosen to be above 60-70% where possible.

DETAILED DESCRIPTION

Current treatment recommendations in RA

RA is a common inflammatory rheumatic disease which causes persistent pain, stiffness and joint damage resulting in significant disability, loss of quality of life and employment. The disease mostly affects women and it appears at the 5th decade of the life.

Based on current guidelines, treatment aims to induce clinical and radiological remission for optimizing physical function, improving the quality of life and work capacity and reducing the risk of comorbidities (Linde, Sorensen et al. 2010; Provan, Semb et al. 2011; van der Heijde 2012; Kavanaugh, Fleischmann et al. 2013; Thiele, Huscher et al. 2013; Radner, Smolen et al. 2014). Current treatment guidelines recommend treatment with csDMARD, in particular, MTX eventually in combination with glucocorticoids to be administered for newly diagnosed RA patients which is applicable in several cases. If the first line MTX therapy does not improve symptoms the next step may be either to switch to another csDMARD (e.g., sulfasalazine, leflunomide, hydroxychloroquine) or to add a biological DMARD (bDMARD) to csDMARD e.g. MTX therapy.

The activity of RA in patients is characterized or quantified by a combined index called Disease Activity Score (DAS28) (Fransen and van Riel 2005). It has been extensively validated for its use in clinical trials in combination with the European League Against Rheumatism (EULAR) response criteria. The DAS28 score is based on the examination of 28 joints.

There are a wide range of measures of disease activity in RA including: examination of the joints for swelling and tenderness, applying a global score of pain and overall status, possibly in the form of questionnaires, measuring blood markers of inflammation (e.g. ESR and CRP), the presence of anti-citrullinated antibodies (ACPA) measurement by X-rays or other, possibly newer imaging techniques such as ultrasound and MRI, however, an RA specific biomarker to determine prognosis and/or treatment response has not characterized yet.

Evaluation of response to a treatment can be made much easier and more objective using the DAS or DAS28. The DAS will provide a number between 0 and 10, indicating how active the RA is at this moment, however, DAS28 serves as a real-time data, it does not reflect to the possible disease outcome.

MDR protein function may also predict patient response to csDMARD treatment as well as biological treatment helping the physician to tailor the therapy. However, switching to biologicals (bDMARDs), including the case when csDMARD treatment is continued in parallel, is often challenging due to unpredictable drug susceptibility and high costs, especially in patients with mildly elevated DAS28 scores.

Earlier results of the present inventors and others by measuring MDR1, MRP1 and BCRP activities with the SOLVO MDQ Kit™, with cell surface staining applied to differentiate $CD3^+$, $CD4^+$ and $CD19^+$ cells suggested that low BCRP and MRP1 MAF activities on $CD3^+$ cells may predict the need to start biological therapy in RA patients whose symptoms do not improve on csDMARD treatment. In this setting DAS28 scores, CRP, IL-6, aCCP and RF values were also recorded. It has been suggested that further decrease of

$CD3^+$ BCRP and increase in $CD3^+$ MRP1 MAF upon follow-up may indicate a good therapeutic response to biological therapy.

To date, although the role of MDR transporter activity in the prediction of response to MTX has been characterized to some extent in RA (see the Background Art chapter above), little is known about the relation of MDR proteins to therapeutic success of biologicals. In contrast to MTX and other csDMARDs, these molecules do not enter the cell, and are therefore not substrates of MDR proteins. However, the cytokines they target are known to interact with these transporters which may provide an indirect effect on these transporter, hitherto largely unknown.

In the prior art clear guidance was not provided as to how to predict the effectiveness of a bDMARD therapy, in particular, anti-TNF therapy, after a csDMARD therapy has been found insufficient before biological therapy is started, in particular not by measuring multidrug transporter activity.

The present inventors have unexpectedly recognized that measuring at least MDR1 or MRP1 activity or MDR1 and MRP1 composite (MAF_C) activity in $CD3^+$ cells in early stage of bDMARD treatment or even before bDMARD treatment a prediction can be made on the effectiveness of bDMARD therapy. Further measurements may also help further the reliability as disclosed herein.

The results of the present inventors indicate that the determination of MAF_C values in $CD3^+$ cells of RA patients is of predictive value prior to the initiation of biological therapy to establish whether the patient will demonstrate sufficient therapeutic response to a biological therapy, in particular anti-TNF therapy or anti-T-cell therapy. Moreover, it has been found that determination of MAF_{MDR} values in $CD3^+$ cells of RA patients is also appropriate to find a threshold which is predictive prior to the initiation of biological therapy to decide whether the patient will be respondent or non-respondent to biological therapy. demonstrate sufficient therapeutic response. A similar tendency could be observed with MRP1 at 0 weeks of the bDMARD treatment, however, in the experiments the distributions of the MAF_{mdr1} values for responders and non-responders could be separated to a lesser extent. Nevertheless, a threshold value can plausibly be found in this type of measurement as well which may predict non-responders e.g. with a sufficient sensitivity or at least positive predictive value.

It is of particular advantage of both MAF_{mdr1} and MAF_C values are determined and both of them is above the given pre-determined threshold whereby the patient can be considered as a non-responder, or not higher than the threshold wherein the patient is an expectable responder to the bDMARD treatment. Additionally, determining MAF_{mdr1} values will contribute to the reliability of the test.

It is of high importance that the present method is appropriate to provide predictors before the start of the bDMARD therapy as in this way applying an expensive therapy, with special danger of side-effects and loss of time by using an ineffective therapy. The results provide an option to decide about further treatment before symptoms reflect the success of the therapy. As in RA the window of opportunity to apply therapy is limited, the invention is useful to find the appropriate treatment in time.

Nevertheless, the method can also be applied in an initial state of the bDMARD therapy for example within the first two months the latest or preferably earlier, e.g. in the first 6 weeks or the first 4 weeks

or the first 2 weeks of the therapy. Certain predictive values can even be more pronounced in this stages.

Thus, if bDMARD therapy has already been started, it makes sense to run the method of the invention as early as possible. Moreover, if the first measurement resulted in a result which allowed to try and initiate bDMARD therapy it is or may be advisable to repeat the measurement at a later though still relatively early stage, e.g. at 4 to 7 weeks of the bDMARD therapy. Measuring MAF_C MAF_{MRP} and MAF_{MDR} values in $CD3^+$ cells at 4 to 7 weeks after the start of biological treatment further improves the accuracy of prediction as to whether adequate therapeutic response may be expected. This knowledge provides help the physician to individually tailor the patient's therapy in a timely manner resulting in positive implications with regards to the cost of treatment and the spectrum of side effects.

The skilled person will understand that MAF values report on transport activity of the substrate applied, and other methods to measure or quantify transport activities in the $CD3^+$ T-lymphocytes can be used in the present invention. Also using a substrate which is transportable by both MRP1 and MDR1 is preferred as when a composite activity value is to be obtained this can be done simply and reliably in a single measurement. The individual activity values for MDR1 and MRP1 can be obtained by using specific inhibitors in this setting.

It is of particular advantage is the substrate is reportable, preferably fluorescent and the results can be obtained and quantified by flow cytometry. This is particularly advantageous as in the present setting the transport activity is to be measured in $CD3^+$ T-lymphocytes only.

The skilled person will also understand that applying the MAF values to report on activities is preferred as this way of quantification of the results reliably reports the activity in a manner which is highly independent from conditions of the measurement, like flow cytometry parameters, and rather sensitive to the cell type which is in line with the nature of this inventive method.

In the present invention when patient samples are measured and responsiveness is assessed no control samples are necessary as pre-determined threshold (cut-off) values are applied.

The utility of csDMARDs and bDMARDs in RA

Today, recommendations for RA treatment are based on the current EULAR guideline (Smolen, Landewe et al. 2017). The most important feature of this guideline is that the decision making should be shared between the patient and the rheumatologist, however, the aim of the therapy is to achieve the treatment goal of remission or at least low disease activity within the time frame of 6 months, at least 50% clinical improvement within 3 months is desirable (Aletaha, Alasti et al. 2016). To achieve this goal Therapy should be started as soon as possible, preferably at the time of diagnosis. Importantly, therapy success should be monitored regularly especially in active disease (every 1-3 moths) and, if there is no improvement, or the goal is not reached by 6 moths, therapy should be modified. If the therapy goals are achieved, the dose of the respective medicine could be declined, or, in complete remission, terminated.

For checking treatment success, the determination of DAS28 levels together with the measurement of rheuma factor (RF), CRP, ACPA and erythrocyte sedimentation rate (ESR) are widely used and a person skilled in the art is able to apply these methods to the present invention.

csDMARDs, especially MTX together with lefluonomide, sulfasalazine and hydroxychloroquine, or in some cases, glucocorticoids, serve as Phase I therapy. Although these agents sometimes have poorly tolerated side effects, MTX should be the first medication in present EULAR recommendations. Importantly, when MTX is contraindicated, or its side effects are poorly tolerated, the patient should be switched to leflunomide, or bDMARD.

Anti-TNF agents (infliximab, trade name: Remicade; etanercept, trade name: Embrel, adalimumab, trade name Flumira, golimumab, trade name: Simponi and certolizumab pegol, trade name: Cimzia) serve as the first-line biological originator (bo)DMARDs, since biosimilar (bs)DMARDs are also available. The first suggested anti-TNF agent is infliximab (IFX), however, it is a chimeric monoclonal, thus, anti-drug antibodies may develop which drastically cuts down the efficiency of this expensive therapy. When first-line anti-TNF agent (bo or bs, respectively) is not successful, another anti-TNF antibody should be used. Importantly, drugs altering immune response should carefully be applied, as in some cases, it may lead infections, moreover, to cancer development (Bongartz, Sutton et al. 2006). Importantly, these Phase II therapies should be given in parallel with csDMARDs or glucocorticoids to make therapy more effective (Nurmohamed and Dijkmans 2008).

When the treatment goal was not reached by using anti-TNF agents, other bDMARDs should be used. These drugs are targeting costimulation (T cell activation, abatacept, trade name: Orencia), causing B cell depletion (rituximab, trade name: Rituxan), blocking IL-6 receptor (tocilizumab, trade name: Actemra, sarilumab, trade name: Kevzara), IL-6 inhibitors (clazakizumab, sirukumab), or blocking IL-1 receptor (anakinra, trade name: Kineret). These treatments, together with tsDMARDS serve as Phase III therapy. tsDMARDs are inhibiting JAK kinases (tofacitinib, trade name: Xeljanz, or baricitinib, trade name: Olumiant). Importantly, Phase II and Phase III therapies should be given in parallel with csDMARDs and it is based on the patient's necessities.

It is then up to the medical personnel guiding the treatment that in case of non-responsiveness for a patient treated or to be treated by anti-TNF therapy or anti T cell activation therapy should be switched to a tsDMARD therapy or at first other bDMARD therapy of different target, like B-cell depletion or IL-6 inhibitors or blocking IL-6 receptor or IL-1 receptor should be applied. Advisably the contemporary EULAR guidance or its national variant should be observed.

MDRs in health and disease

Transport of compounds between the intra- and extracellular compartments is an essential physiologic phenomenon. For this, several transmembrane pumps evolved, showing strong sequence homology between different species.

The core functional unit of ABC transporters contains two membrane-spanning domains, each of which typically contains 6 transmembrane (TM) helicases. In the intracellular compartment, 2 nucleotide-

binding domains (NBDs) are localized which contain Walker A and Walker B domains, that are necessary for ATP binding and hydrolysis (Deeley, Westlake et al. 2006; Silva, Almeida et al. 2015),

As ABC transporters originally involved in the detoxification of the organism, the members of the ABC transporter family are expressed on a wide variety of tissues and organs, like intestine, lung, liver, testes, placenta, skeletal and cardiac muscle and on the endothelial surface of the blood-brain barrier (Flens, Zaman et al. 1996; St-Pierre, Serrano et al. 2000; Wijnholds, deLange et al. 2000; Mercier, Masseguin et al. 2004; Castilho-Martins, Canuto et al. 2015). The majority of ABC transporters are expressed on the apical and basolateral surface of polarized cells (Hipfner, Gauldie et al. 1994; Evers, Zaman et al. 1996), however, in special cases, i.e. in drug selected cell lines, MDR1 is shown to be localized in the Golgi complex as well (Cole, Bhardwaj et al. 1992).

Beside their crucial role in the maintenance of homeostasis, ABC transporters are also involved in the phenomenon, called multidrug resistance (MDR), which makes therapy ineffective by removing drugs from target cells. Since MDR is the principal mechanism by which many tumours develop resistance to chemotherapeutics or immunosuppressant drugs administered in different types of leukaemia, solid tumours and autoimmune diseases and to patients who underwent transplantation. Conventional anticancer drugs (doxorubicin, gefitinib, irinotecan, methotrexate, paclitaxel, tamoxiphen, topotecan, etc.) are substrates of MDR transporters. Moreover, MDR transporters play distinct role in the fine tuning of the immune response.

qRT-PCR, immunohistochemistry and Western blots are the most frequently used methods to determine the MDR transporter status in clinical samples. More recently, mass spectrometry based methods have been described to quantify transporter expression (Prasad, Lai et al. 2013). On the other hand, several polymorphisms affecting transporter functions have been reported (Porcelli, Lemos et al. 2009; Lee, Chau et al. 2010). Therefore, relevance of even protein levels as solitary pieces of data is questionable. Some of the genetic variants affect transporter trafficking, and, thus, FACS-based determination of cell surface expression of MDR transporters is a significant progress (Damiani, Tiribelli et al. 2006). However, antibodies recognizing the extracellular MDR1 (Georges, Tsuruo et al. 1993; Vasudevan, Tsuruo et al. 1998) and BCRP (Telbisz, Hegedus et al. 2012) epitopes are conformation sensitive, making their determination challenging.

Such methods can be applied in and to the present invention, however, adaptation is needed. Using fluorescent substrates and flow cytometry and quantifying the results as MAF values as shown herein is advantageous due to reliability and simplicity. Using a calcein ester which is substrate of both MDR1 and MRP1 and which is trapped in the $CD3^+$ T-lymphocytes once cleaved are of particular advantage as explained more specifically below.

The determination of transporter activities

The transporter activity can be among other measured by a kit designed for functional quantitative measurement of drug resistance in live cells. The procedure is preferably fast, sensitive, and quantitative. The procedure should preferably measure the drug transport activity of at least two subfamilies of multidrug resistance proteins: MDR1 and MRP1. MDR1 and MRP1 are ATP-dependent

trans-membrane proteins that remove hydrophobic xenobiotic compounds (typically environmental toxins) from the cell. A preferred kit utilizes calcein-AM, a non-fluorescent hydrophobic compound that enters all cells by passive diffusion via the plasmamembrane. Calcein-AM is an excellent substrate for targeted extrusion by multi-drug transporters. If MDR1 and MRP1 are active, the hydrophobic calcein-AM will be removed intact before it can be hydrolyzed. If MDR1 and MRP1 are not active, enzymatic cleavage of the calcein-AM by endogenous esterases results in the fluorescent hydrophilic free-acid, calcein, which is retained within the cytoplasm. Normal, drug sensitive cells will fluoresce when exposed to calcein-AM. The degree of fluorescence observed in test cells is inversely proportional to MDR1 and MRP1 activity. An example for such a kit is The SOLVO MDQ Kit which has CE-IVD certification (available from MDQuest, Szeged, Hungary).

Quantitation of this fluorescence is possible through the development of the MDR Activity Factor (MAF). The dye efflux activity of the MDR transporter is measured as the difference between the amount of the dye accumulated in the presence and absence of inhibitors.

The fluorescence measurement in the presence of an inhibitor specific to both MDR1 and MRP1 constitutes the maximal potential fluorescence with the given cell population when the multidrug transporters are rendered nonfunctional. This represents a standardization method, which eliminates unknown cell type-specific variables that influence cellular calcein accumulation, such as esterase activity, cell size, etc. This, in turn, allows for intra- and interlaboratory comparison of test results and MAF values. The transport activity of MDR1 and MRP1 can be easily distinguished with inhibitors specific to one of these proteins.

Inhibitors, which are known to those skilled in the art, preferably includeverapamil, and also included, but are not limited to, e.g., verapamil, indomethacin, oligomycin, or cyclosporin.

The kit has been optimized for and its preferred use is in flow cytometry, but can be adapted for use in other cell-based assay formats such as fluorescence microscopy, spectrophotometry, or 96 well plate assays. If these applications are utilized it is necessary to consider the following:

- Heterogeneous cell populations accumulate calcein at different rates, which cannot be resolved by fluorometry (cuvette or plate reader).
- Homogeneous cell population can be easily tested in the above-mentioned formats.
- For consistency and reproducibility, adequate mixing of cell suspensions and temperature control are necessary.
- Protocol adaptation for other formats will be necessary.

A more detailed description of a particular kit is provided in the Examples.

Practical aspects in carrying out transport activity measurements

In particular, for determining transporter functions in RA patients with commercially available detection kits, application of any internal and external controls are not required, since the cut-off values for each transporters in all time points are clearly defined.

For testing the performance of the preferred kit, cell lines overexpressing of MDR1, MRP1 and BCRP could be used.

For checking the flow cytometry equipment, commercially available fluorescent microbeads are recommended.

In one embodiment of the method of detecting multi-drug resistance in a biological sample, the control cells can be a portion of the biological sample itself, the method further including exposing the control cells to an inhibitor of multi-drug resistance. By using portions of the same biological sample, or by controlling the temporal sequence by which the components are added, the control acts as an internal, or "self", control.

The MAF values of healthy adults on $CD3^+$ T lymphocytes have already been determined according to the CLSI guideline C28-A2. In that study, 120 healthy adults (age between 18 and 74 years) were enrolled. In parallel with measuring MAF values, CD4/CD8 ratio, blood cell count, liver and kidney function were determined. For performing transporter activity measurements, 6 mis of K_3EDTA anticoagulated peripheral blood samples were collected from each individual. PBMCs were separated by using Ficoll Flistopaque density gradient centrifugation according to the manufacturer's instructions. The applied assay was performed as it was described by the instructions for users. After running assay, $CD3^+$ cells were labelled with PerCP or FITC conjugated anti-CD3 antibodies. The measurements were carried out on a BD FACSCalibur flow cytometer equipped with 488 nm argon and 635 nm red diode lasers. The calculation of MAF values were performed as it was described previously. Importantly, no statistical significance was determined between men (n=62) and women (n=58). Interestingly, the age of the individual had no impact on MAF values in case of MRP1 and BCRP, however, in case of MDR1 and MAF_C values, a negative correlation was determined between the values and the age of the studied individuals. Based on the previous facts, the cut-off values for transporter activities, which can be considered as an average for the healthy European adult population, are the following: MAF_C: 16.5; **MAFMDRI-** 12.9; **MAF** $_{MRP1}$**:** 2.5; **MAF**$_B$**c**$_{RP}$**:** 3.4.

Finding an activity threshold value to distinguish between responders and non-responders.

The receiver operator curve (ROC) is a fundamental tool for diagnostic test evaluation. When the results of a diagnostic test are considered to discriminate between two populations (eg. responders versus non-responders), a perfect separation between the two groups is rarely observed. For every possible cut-off point selected to discriminate between the two populations, there will be some cases with the responder status correctly classified as responder (True Positive), but some responders will be classified into the non-responder group (False Negative). On the other hand, the majority of non-responders will be correctly classified as non-responders (True Negative), but some will be classified as responders (False Positive). In a ROC curve the true positive rate (Sensitivity: calculated as the True Positive / (True Positive + False Negative)) is plotted against the false positive rate (100-Specificity; wherein specificity is calculated as the False Positive / (False Positive + True Negative), i.e. 100-Specificity is the True Negative / (False Positive + True Negative)), demonstrating different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (Zweig and Campbell 1993). While the ROC analysis is widely applied,

the skilled person will understand that any means preferably mathematical statistical means for finding a threshold to separate the two overlapping distributions may be applied in the present invention.

Our ROC analysis revealed that the assessment of multidrug activity of peripheral blood lymphocytes carries predictive value for response to bDMARD treatment in RA patients at the start of therapy. Patients with MAF values above the cut-off thresholds are likely to be Non-responders to treatment. Of note, these cut-off values are all below the respective reference ranges in healthy individuals established in our earlier study.

Additional criteria to distinguish between responders and non-responders

MDR-ABC transporters transport a variety of endogenic molecules, such as cytokines and chemokines that play an important role in the pathogenesis of RA and therefore may be used as biomarkers to monitor disease progression in RA. They may also be used as a predictive tool to establish responsiveness to biological therapy. In this multicenter clinical trial, we aimed to assess the predictive value of flow-cytometry based multidrug resistance activity measurement of three clinically relevant MDR proteins (MDR1, MRP1, BCRP) for biological therapeutic response in rheumatoid arthritis in $CD3^+$ and $CD19^+$ lymphocytes before as well as 4 to 6 and 12 weeks after the initiation of biological therapy.

EXAMPLES

METHODS

Measurement of MDR1 and MRP1 activities by the calcein assay

Quantitative measurement of MDR1 and MRP1 activities in viable cells is carried out using the calcein-assay technology (see US5872014A). As a preferred kit the *SOLVO MDQ Kit was used.* This method has several advantages against other fluorescent dye accumulation tests: it is quick, quantitative, selective for MDR1 and MRP1 transporters and it has validated internal standard. This assay utilizes the fluorogenic dye calcein-acetoxymethyl ester (calcein-AM) a hydrophobic compound that readily penetrates the cell membrane. After entering into the living cell, the non-fluorescent calcein-AM is rapidly hydrolysed by endogenous esterases to form a highly fluorescent free acid derivative of the dye which becomes trapped in the cytoplasm due to its high hydrophilicity. Another advantage of calcein is the relative insensitivity to changes of various cellular parameters, including intracellular pH, $Ca^{2+}$and Mg^{2+}concentrations.

As calcein-AM is an excellent substrate of both MDR1 and MRP1, activity of these efflux transporters results in lower cellular accumulation of the fluorescent calcein. Consequently, the more MDR proteins are active in the cell membrane, the less calcein is accumulated intracellularly. In MDR expressing cells, the addition of selective inhibitors of MDR1 and MRP1 blocks the dye exclusion activity of the relevant transporter and increases calcein accumulation in the cells. In the absence of significant MDR transporter activity, the lack of transporter mediated efflux means that the net calcein accumulation is faster in the cells, which, in turn, is not influenced by the presence of an MDR transporter inhibitor or substrate.

Respective activities of MDRs are reflected by the difference between the amount of calcein accumulated in the presence or absence of selective inhibitors. When calculating the MAF values, this

accumulation difference is normalized to the dye uptake measured in the presence or the absence of the inhibitor and the results of the assay are expressed in MDR activity factor (MAF) values. Thus, the result of the test is independent from factors influencing the cellular accumulation of Calcein other than the activity of the multidrug transporters. Such factors involve the difference in cellular properties (membrane lipid composition, intracellular esterase activity, cell size, cell surface, etc) and the methodological differences (i.e.: using different equipment, amplification and individual variables). Since the influence of these non-MDR transporter mediated factors are reduced by the normalization approach mentioned above, this facilitate intra- and interlaboratory comparison of MAF values.

Selective inhibitors can be used to distinguish between the transport activity of MDR1 and MRP1. The pan-MDRl/MRPl inhibitor blocks both MDR1 and MRP1 mediated dye effluxes, providing dye accumulation rate that can be used for standardization, while MRP1 blocker helps to determine MDR1 and MRP1 activity. After a short, simple calculation, separate measurement of multidrug resistance for both MDR1 and MRP1 activity can be obtained.

BCRP activity is measured using a similar principle: intracellular accumulation of the fluorescent BCRP specific probe substrate is measured in the presence or the absence of selective BCRP inhibitor. Flowever, in this case, the BCRP specific probe substrate is direct fluorescent and does not require cleavage by intracellular enzymes.

It is possible to perform MDR activity measurement on a dedicated cell population of interest by labelling them with fluorochrome-conjugated antibodies after running assay procedure. The assay-compatible fluorochromes are listed in ***Table 1.***

Table 1. Examples for assay compatible fluorescent conjugates

Transporter(s)	Dye / substrate	Channel	Compatible fluorochrome
MDR1, MRP1	calcein	~515 nm	PerCP; PerCP-5.5
BCRP	mitoxanthrone	~684 nm	FITC, PE

Use of the SOLVO MDQ Kit™

The SOLVO MDQ Kit™ was used strictly following the manufacturer's instructions. PBMCs were loaded with fluorescent MDR activity reporter substrates (Calcein-AM for MDR1 and MRP1, em: 515 nm and mitoxantrone for BCRP, em: 684 nm, respectively) and treated with MDR protein specific inhibitors (verapamil for MDR1 and MRP1, indomethacin for MRP1 and K0134 for BCRP, respectively) to obtain multidrug activity factor (MAF) values.

Cell surface staining was applied to select $CD3^+$ T lymphocytes using anti-human CD3-PerCP monoclonal antibodies in case of Calcein-AM stained cells and anti-human CD3-FITC monoclonal antibodies in case of mitoxantrone stained cells according to the manufacturer's instructions.

MAF values were calculated from the difference between the geometric mean fluorescent intensity (MFI) of cells with and without the specific inhibitors, respectively.

MAF_C (composite MAF of MRP1 and MDR1) = $100 \times (F_{max} - F_o) / F_{max}$

MAFMRPI (MAF of MRP1) = $100 \times (F_{MRPI} - F_o) / F_{max}$

MAF_{MDR1} (MAF of MDR1) = MAF_C - MAF_{MRP1}

MAF_{BCRP} (MAF of BCRP) = 100 x (F_{MX} - F_o) / F_{MX}

F_{max}/ F_{MX}: Calcein/mitoxantrone fluorescence with verapamil or K0134, respectively

F_o: fluorescence without inhibitor

F_{mrp1}: Calcein fluorescence with indomethacin

Patient Recruitment

39 RA patients were recruited at the outpatient clinics of the Department of Rheumatology, University of Debrecen, Flungary and the Department of Rheumatology and Clinical Immunology, Charite, Berlin, Germany. Patients were sampled before the start of biological treatment as well as between 4 and 7 weeks and at 12 weeks of treatment. DAS28 and CRP values were also recorded in parallel with MAF determination. Patients were regarded as non-responders (n = 12) if DAS28 values showed a decrease of less than 25% between the start of biologicals and at 12 weeks of treatment. Patient characteristics as well as details of the therapy received are included in Table 2. Healthy controls (n = 35) were sampled at the Department of Rheumatology, University of Debrecen, Hungary on a single occasion. They had a negative history of autoimmune disorders including RA and a negative status upon physical examination as well as no infectious symptoms within three weeks before sampling.

Exclusion criteria for all participants included chronic infectious diseases requiring systemic treatment, autoimmune diseases other than RA, immunodeficiencies, allergic diseases and hematological malignancies or solid tumors, age below 18 years. Written informed consent was obtained from all participants and the study adhered to the tenets of the most recent revision of the Declaration of Helsinki.

Peripheral Blood Mononuclear Cell (PBMC) isolation

6 mis of K_3EDTA anticoagulated peripheral blood sample was collected. PBMCs were separated by density gradient centrifugation using Ficoll Histopaque-1077 (Cat. No: H8889, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Flow Cytometry

Measurements were conducted on a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) equipped with 488 nm and 635 nm lasers or on a Miltenyi MACSQuant flow cytometer, equipped with 405 nm, 488 nm and 638 nm lasers, respectively.

The SOLVO MDQ Kit was used strictly following the manufacturer's instructions. In this assay, fluorescent reporter substrates are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of specific inhibitors, allowing for quantitative, standardized assessment. PBMCs were loaded with fluorescent MDR activity reporter substrates (Calcein-AM for MDR1 and MRP1, em: 515 nm and mitoxantrone for BCRP, em: 684 nm, respectively) and treated with MDR protein specific inhibitors (verapamil for MDR1 and MRP1, indomethacin for MRP1 and K0134 for BCRP, respectively) to obtain multidrug activity factor (MAF) values.

Cell surface staining was applied to select $CD3^+$ and $CD19^+$ cells using anti-human CD3-PerCP and CD19-PE monoclonal antibodies (Cat. No: 345766 and 345789, respectively, both BD Biosciences) in case of

Calcein-AM stained cells and anti-human CD3-FITC and CD19-PE monoclonal antibodies (Cat. No: 345764 and 345789, respectively, both BD Biosciences) in case of mitoxantrone stained cells according to the manufacturer's instructions. Assay-compatible fluorochromes are listed in Table 1.

Results

ROC analysis was performed to evaluate the predictive value of **MAF** for response to treatment in RA patients at the start of biological therapy and at 6wk. Cut-off thresholds were calculated for **MAF** values with ROCs of adequate p and AUC values (Figure 3). Patients with **MAF** values above the respective cut-off thresholds are likely to be Non-responders to treatment (**MAF_C** of $CD3^+$ cells at Owk: p = 0.043, AUC = 0.68; **MAFc** of $CD3^+$ cells at 6wk: p = 0.033, AUC = 0.72; MAF_{MDR1} on $CD3^+$ cells at 6wk: p = 0.048, AUC = 0.70; MAF_{MRP1} on $CD3^+$ cells at 6wk: p = 0.049, AUC = 0.69).

In our multicenter clinical trial, 39 RA patients were enrolled. For determining the functional activities of MDR1, MRP1 and BCRP, 6 mis of K_3EDTA anticoagulated blood peripheral blood samples were collected. PBMCs were separated by using Ficoll Flistopaque density gradient centrifugation according to the manufacturer's instructions. SOLVO MDQ Kit™ assay was performed as it is described in the instructions for users. After performing the assay, $CD3^+$T lymphocytes were labelled with PerCP or FITC - conjugated anti-CD3 antibodies for 30 minutes. After removing unbound antibodies, transporter activities were determined on $CD3^+$T lymphocytes by flow cytometry.

Clinical characteristics of patients are indicated in Table 2.

Table 2. Clinical characteristics of Responder and Non-responder RA patients as well as healthy controls. Data are expressed as median (IQR) for continuous variables and as number (percentage) for categorical variables. MTX - methotrexate

	Healthy controls (n = 35)	**Responder (n = 27)**	**Non-responder (n = 12)**
Age (years)	54 (42-62)	56 (49-61)	51 (39-61)
Gender (male/female)	4/31	2/25	1/11
RA duration (years)	-	10 (5-14)	8.5 (5-15)
No. of patients receiving MTX	-	15 (56%)	6 (50%)
No. of patients receiving prednisolone	-	9 (33%)	5 (42%)
No. of patients receiving adalimumab	-	2 (7%)	1 (8%)
No. of patients receiving certolizumab pegol	-	5 (19%)	3 (25%)
No. of patients receiving etanercept	-	7 (26%)	3 (25%)
No. of patients receiving abatacept	-	13 (48%)	5 (42%)

Importantly, in parallel with collecting blood samples at the time of diagnosis (0 week) and during regular checkups (2, 6 and 12 weeks, respectively) DAS28 score was determined and the routinely used inflammatory markers (RF, CRP, ESR, ACPA) were also measured from peripheral blood. bDMARD treatment responsivity was determined on the alterations of DAS28 scores.

Regarding to treatment success (Figure 1), the baseline DAS28 value was remarkably higher (average: 5.94; 5.11-6.17) as compared with non-responders (average: 4.65; 2.79-4.45). In case of responders, significant DAS28 down regulation was detected 6 weeks after starting bDMARD as compared with 0 week values (3.71 vs 5.94) which became more pronounced at 12 weeks checkup (average: 3.00). In contrary with responders, bDMARD treatment had no impact on DAS28 values neither 6, nor 12 weeks after starting therapy (3.93 and 3.90, respectively).

ROC analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6wk. Cut-off thresholds were calculated for MAF values with ROCs of adequate p and AUC values (Figure 3, Figure 4). Patients with MAF values above the respective cut-off thresholds are likely to be Non-responders to treatment (MAF_C of $CD3^+$T lymphocytes at Owk: p = 0.043, AUC = 0.68; cut-off: 21.3; MAF_c of $CD3^+$T lymphocytes at 6wk: p = 0.033, AUC = 0.72, cut-off: 12.3; MAF_{MDR}I on $CD3^+$T lymphocytes cells at 6wk: p = 0.048, AUC = 0.70, cut-off: 13.9; MAF_{MRP}I on $CD3^+$T lymphocytes at 6wk: p = 0.049, AUC = 0.69, cut-off: 6.0). In case of MAF_{MDR}I of $CD3^+$ T lymphocytes at 0 wk, the cut-off value is 17.4, however, based on the low patient number, statistical significance was not detected (p=0.24).

MAF values of $CD3^+$T lymphocytes from RA patients showed the following values: at the time of diagnosis, MAF_Cvalues of responders were almost the same as compared with healthy individuals (18.9 vs 18.3), however, in case of non-responders, MAF_C values on $CD3^+$T lymphocytes were significantly upregulated as compared with controls (23.5 vs 18.3). During bDMARD treatment in case of responders, a slight down regulation was detected 6 weeks after starting therapy, however, in later time points, MAF_Cvalue did not showed any alterations as compared with control samples and values at the time of diagnosis. Importantly, in case of responders, average MAF_Cvalues were below the cut-off values at the time of diagnosis and 6 weeks after starting bDMARD treatment. In contrary with responders, MAF_Cvalues of non-responders were significantly higher as compared with healthy controls at the time of diagnosis (23.5 vs 18.3). As same as responders, bDMARD treatment had no impact on MAF_Cvalues, however, MAF_c of $CD3^+$T lymphocytes during bDMARD treatment were significantly higher as compared with healthy counterparts.

Although in case of MAF_{mdr1} cut-off value statistical significance was not detected at the time of diagnosis (17.4; 0 weeks), its prognostic value is still high, in particular together with the 6 weeks cut-off value data (13.9). At the time of diagnosis, responder values did not showed any alterations as compared with controls, however, MAF_{mdr1} values of non-responders were significantly above the control data (19.1 vs 14.6). Prolonged bDMARD treatment had no significant impact on MAF_{MDR1}values of responders. In case of non-responders, mild down regulation was detected after starting bDMARD treatment as compared with values at the time of diagnosis.

MAF_{mrp1} values has strong prognostic value 6 weeks after starting bDMARD treatment. At the time of diagnosis (0 weeks?), mild upregulation was detected in RA patients as compared with healthy controls. 6 weeks after starting bDMARD treatment, a mild down regulation was detected as compared with 0 weeks value. Importantly, opposed to responders, a significant upregulation was detected in non-responders (2.2 vs 8.4).

Results are summarized in Table 3.

Table 3 Activity of various MDR transporters on $CD3^+$ and $CD19^+$ cells in RA patients and healthy controls. Data are expressed as median (IQR), $p < 0.05$ [a] vs Control, [b] vs Responder, [c] vs 0 wk value. MAF_C- composite multidrug activity factor (of MRP1 and MDR1 activity), MAF_{MRP}I- multidrug activity factor of MRP1, MAF_{MDR}I - multidrug activity factor of MDR1, MAF_{BCRP} - multidrug activity factor of BCRP

		0 wk		6 wk		12 wk	
	Control	**Responder**	**Non-responder**	**Responder**	**Non-responder**	**Responder**	**Non-responder**
DAS28	-	5.94 (5.11-6.17)	4.65[b] (3.33-5.23)	3.71[c] (2.79-4.45)	3.93 (3.14-4.50)	3.00[c] (2.23-3.67)	3.90[b] (2.81-4.90)
CRP	-	11.1 (2.6-16.6)	8.4 (1.4-15.1)	4.4 (1.3-7.9)	4.4 (1.5-10.4)	3.7 (2.1-5.6)	7.5 (2.7-11.6)
CD3 MAF_C	18.3 (14.7-22.9)	18.9 (14.0-25.2)	23.5[b] (17.1-33.7)	17.1 (12.3-22.6)	22.7[b] (16.7-29.2)	18.3 (15.7-24.2)	25.2 (15.9-30.7)
CD3 MAF_{MRP1}	3.1 (1.2-5.7)	4.8 (0.0-8.0)	5.7 (2.2-8.0)	2.2 (0.0-7.9)	8.4[b] (2.1-11.3)	5.7 (3.7-8.5)	7.7[a] (4.0-11.6)
CD3 MAF_{MDR1}	14.6 (12.5-18.1)	12.9 (11.0-16.7)	19.1[b] (11.2-24.0)	12.4 (11.2-15.4)	15.8[b] (14.3-18.7)	12.5 (9.2-17.5)	13.6 (6.0-20.0)
CD3 MAF_{BCRP}	2.5 (0.8-5.7)	3.1 (0.0-4.4)	5.0 (2.0-8.0)	2.0 (0.0-5.5)	3.9 (2.5-10.7)	1.4 (0.0-4.3)	4.5 (1.8-5.8)
CD19 MAF_C	12.8 (8.9-17.9)	15.1 (8.1-22.1)	20.6 (13.5-31.0)	13.2 (9.3-20.4)	17.6 (11.4-27.2)	17.4 (13.1-22.3)	17.6 (9.2-25.9)
CD19 MAF_{MRP1}	2.2 (0.0-6.3)	0.9 (0.0-7.7)	4.4 (0.0-5.8)	0.6 (0.0-5.1)	6.8[b] (0.5-9.6)	3.2 (0.3-6.8)	5.1 (1.9-10.9)
CD19 MAF_{MDR1}	9.9 (8.0-14.0)	11.1 (6.0-16.3)	15.7 (8.4-25.4)	11.4 (5.3-14.8)	13.6 (8.6-17.7)	14.0 (7.1-17.7)	8.8 (1.9-15.7)
CD19 MAF_{BCRP}	3.8 (1.0-6.3)	3.1 (0.7-7.0)	4.5 (0.0-11.0)	2.7 (0.0-5.2)	5.0 (3.1-8.4)	2.9 (1.3-5.1)	3.0 (1.8-3.7)

Case studies

Examples from our own clinical trial for predicting patient's response to bDMARD treatment:

Patient 1: 53 years old women who received abatacept (T cell blocking agent). Her DAS28 values showed gradient down regulation during the monitored period (5.94; 5.22; 4.11; 3.36, respectively). Her **MAFc** and MAF_{mdr1} values at the time of diagnosis were 4.2 and 4.1, respectively, which are remarkably below the cut-off value (21.3 and 17.4, respectively). 6 weeks after starting abatacept treatment, her prognostic values are the following: $\mathbf{MAF_C}$: 20.1 (cut-off 20.3); MAF_{MRP}I: 7.8 (cut-off 6.0) and MAF_{MDR}I 12.3 (cut-off:13.9). During all chekups remarkable improvement was recorded regarding to her disease status.

Patient 2: 61 years old female patient with etanercept (anti-TNF) treatment. Her DAS28 values showed significant decrease during the clinical trial (5.59; 4.58; 2.3; 1.9, respectively). Her baseline MAF_C value was 20.5, which is below the cut-off value. 6 weeks after starting anti-TNF therapy, her MAF_C value was drastically declined (11.5 vs 20.5) which also suggests favorable treatment response. In the same time point, her MAF_{MRP}I and her MAF_{MDRi} values were also significantly below the respective reference values (0.0 and 11.5, respectively). Importantly, her physician also recorded favorable treatment response during the whole study period.

In contrary with Patients 1 and 2, **Patient 3** (68 years old woman) showed poor response to abatacept treatment. Regarding to her DAS28 values, no difference was detected during the whole study period (3.06; 3.06; 3.03; 3.03, respectively). Her baseline MAF_C value was remarkably over the cut-off value (22.8 vs 21.3). 6 weeks after starting abatacept treatment, her MAF_C value showed a more robust elevation (24.9) which is over the cut-off value. The same tendency was detected in case of MAF_{mdr1}, the 8.3 MAF_{MDRi} value increased to 14.3 which suggests unfavorable treatment outcome. In accordance with the previously mentioned values, her MAF_{MRP1} was also dramatically elevated as compared with the cut-off value (10.6 vs 6.0). In accordance with transporter activity data, no improvement was detected reading to her disease status, thus a tsDMARD treatment would highly be recommended to her.

INDUSTRIAL APPLICABILITY

The invention is useful to provide predictors before the start of the bDMARD therapy and thereby an option to decide about further treatment before symptoms reflect the success of the therapy. As in RA the window of opportunity to apply therapy is limited, the invention is useful to find the appropriate treatment in time.

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CLAIMS

1. An *in vitro* diagnostic method for assessing the responsiveness of a sDMARD *(therapies?)* (synthetic disease-modifying antirheumatic drug) treated RA patient to bDMARD therapy (biological synthetic disease-modifying antirheumatic drug therapy), wherein preferably the patient is in need of a switch or modification of the sDMARD therapy, said method comprising the steps of

- providing a biological sample of said sDMARD treated RA patient, said sample comprising $CD3^+$ T-lymphocytes from said patient,
- obtaining one or more transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD treated RA patient, before or at an initial phase of a bDMARD therapy, by using one or more substrate(s) of MDR1, MRP1 or of both MDR1 and MRP1,
- comparing the one or more transporter activity value(s) with one or more pre-determined threshold transporter activity level(s),

wherein each pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters and which has been determined using the same one or more substrates,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of said one or more transporter activity value(s) measured is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of each transporter activity value is not higher than said threshold level,

wherein preferably said bDMARD therapy is selected from the group consisting of

- anti-TNF therapy,
-T-cell activation inhibitor therapy,
- B lymphocyte depletion therapy,
-anti-IL6 therapy, preferably
- anti-TNF therapy and
-T-cell activation inhibitor therapy,
- wherein preferably the sDMARD therapy is a preferably csDMARD therapy.

2. The *in vitro* method of any of claim 1, wherein

the transporter activity value is obtained by measuring transport activity of one or more multidrug transporters comprising at least MDR1 in the $CD3^+$ T-lymphocytes of said sDMARD treated RA patient before a bDMARD therapy or in an initial phase thereof, by using an MDR1 substrate, and

the MDR1 activity value is compared with a pre-determined threshold transporter activity level, wherein said pre-determined threshold transporter activity level has been determined using the same MDR1 substrate,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of the MDR1 activity value is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of the MDR1 activity value is not higher than said threshold level.

3. The *in vitro* method of claim 2, wherein
the transporter activity value is obtained by measuring transport activity of one or more multidrug transporters comprising MDR1 and MRP1, in the $CD3^+$ T-lymphocytes of said sDMARD treated RA patient before a bDMARD therapy or in an initial phase thereof, and
the transporter activity value is compared with a pre-determined threshold transporter activity level, wherein said pre-determined threshold transporter activity level has been determined using the same substrate,
- considering said RA patient as a non-responder to the bDMARD therapy when the level of the MDR1 and MRP1 transporter activity value is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of the MDR1 and MRP1 transporter activity value is not higher than said threshold level.

4. The *in vitro* method of claim 3 wherein
wherein said MDR1 and MRP1 activity is measured with a substrate of both MDR1 and MRP1, the transporter activity comprising activities of both MDR1 and MRP1 and the two transporters are not differentiated by inhibition, whereby a composite transporter MDR1-MRP1 activity value is obtained.

5. The *in vitro* method of any of claims 1 to 4 wherein said threshold transporter activity level has been determined by using the one or more substrates by
measuring or quantifying the transport activity of said one or more multidrug transporters in the $CD3^+$ T-lymphocytes in a reference patient group known to be responder to the bDMARD therapy and a further reference patient group known to be non-responder to the bDMARD therapy, and
the transport activity values measured in the responder and non- responder patient groups are statistically analysed as distributions to find a threshold level which differentiates between responder transport activity values and non-responder transport activity values.

6. The *in vitro* diagnostic method of any of claims 1 to 5 wherein measuring the transporter activity comprises
- contacting at least the $CD3^+$ T-lymphocytes in the biological sample with the one or more transporter substrate(s), said substrate being a derivative of a detectable fluorescent compound, and wherein said derivative is taken up by at least the $CD3^+$ T-lymphocytes and is hydrolyzed into said fluorescent compound in the cells, wherein said fluorescent compound is not transportable by MDR1 or MRP1 or

by neither MDR1 nor MRP1 or transportable to a significantly lesser extent than the ester derivative, and

- measuring fluorescence in the $CD3^+$ T-lymphocytes,

- obtaining the transport activity value from the fluorescence in the $CD3^+$ T-lymphocytes,

wherein preferably

at least MDR1 activity is measured and/or

at least a composite transporter activity is measured, with a substrate of both MDR1 and MRP1, wherein the two transporters are also not differentiated by inhibition and a composite activity value is obtained.

7. The *in vitro* diagnostic method of any of claims 1 to 6 wherein the substrate is a detectable fluorescent ester compound.

8. The *in vitro* diagnostic method of claim 7 wherein

measuring transport activity is based on the difference between the fluorescence of the detectable fluorescent compound in the cells measured in the presence of an inhibitor of the multidrug transporters and the fluorescence measured in absence of said inhibitor,

wherein preferably measurement is carried out by flow cytometry.

9. The *in vitro* diagnostic method of any of claims 7 to 8 wherein the biological sample is peripheral blood and

the substrate is calcein ester and the transporter activity (value) threshold level is calculated as an MDR activity factor for MDR1 (preferably MAF_{mdr1}) and said threshold is between 15 to 19, preferably 16 to 19 or 16 to 18, more preferably between 17 to 19 or 17 to 18, preferably about 17.4, or

the substrate is a detectable fluorescent ester compound the transporter activity (value) threshold level is calculated as an MDR activity factor for MDR1 (preferably MAF_{mdr1}) and said threshold is equivalent with the one obtained for calcein.

10. The *in vitro* diagnostic method of any of claims 7 to 9 wherein the biological sample is peripheral blood and the MDR1 substrate is calcein ester and the transporter activity (value) threshold level is calculated as a composite MDR activity factor for MDR1 and MRP1 (preferably MAF_c) and said threshold is between 19 to 23, preferably 20 to 22 more preferably between 20.8 to 21.8, preferably about 21.3, or

the substrate is a detectable fluorescent ester compound the transporter activity (value) threshold level is calculated as an MDR activity factor for MDR1 (preferably MAF_{mdr1}) and said threshold is equivalent with the one obtained for calcein.

11. The diagnostic method of any of claims 1 to 8 wherein the initial phase of the bDMARD therapy is no longer than 8 weeks, preferably 7 weeks or 6 weeks, highly preferably 4 weeks, even more preferably 2 weeks.

12. The *in vitro* diagnostic method of any of claims 1 to 11, wherein said sDMARD-treated RA patient has been also treated by bDMARD therapy,
said method additionally comprising
- providing a further biological sample of said sDMARD-treated RA patient between weeks 4 and 7 of the bDMARD therapy,
- obtaining one or more further transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient between weeks 4 and 7 of the bDMARD therapy, by using one or more substrate(s) of MDR1, MRP1 or both MDR1 and MRP1,
- comparing one or more further transporter activity value(s) with one or more pre-determined threshold transporter activity level(s),
wherein each pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters and which has been determined using the same one or more substrates, as defined above,
- considering said RA patient as a non-responder to the biological therapy wherein the level of each transporter activity value measured is above said each threshold level, and
- considering said RA patient as a responder to the biological therapy wherein the level of each transporter activity value is not higher than said each threshold level
wherein preferably the bDMARD therapy is a bDMARD therapy as defined in claim 4, and
wherein preferably measuring or quantifying transporter activity of transporter protein is carried out as defined in claims 1 to 10, preferably in claim 6 or in claims 9 or 10, and
wherein preferably the substrate is a substrate of both MDR1 and MRP1 and highly preferably the substrate is a calcein ester.

13. The *in vitro* diagnostic method of claim 12 wherein
- at least the MDR1 activity is measured, and the transporter activity value is MAF_{mdr1} value, and
said RA patient is considered as a non-responder to biological therapy wherein if the substrate is a calcein ester then the level of MAF_{MDR1} is above a MAF_{mdr1} threshold from 12 to 15, preferably above 13.9 or
if the substrate is a detectable fluorescent ester compound the MAF_{mdr1} threshold value is calculated as an MDR activity factor for MDR1 (preferably MAF_{mdr1}) and said threshold is equivalent with the one obtained for calcein;
- at least the MRP1 activity is measured, and the transporter activity value is MAF_{mrp1} value, and

said RA patient is considered as a non-responder to biological therapy wherein the level of MAF_{MDRP1}.is above a MAF_{mrp1} threshold from 5 to 7, preferably above 6.0 or

if the substrate is a detectable fluorescent ester compound the MAF_{mrp1} threshold value is calculated as an MDR activity factor for MRP1 (preferably MAF_{mrp1}) and said threshold is equivalent with the one obtained for calcein

- at least a composite activity is measured, and the transporter activity value is MAF_C value, and

said RA patient is considered as a non-responder to biological therapy wherein the level of MAF_c.is above a MAF_C threshold from 18 to 22, preferably above 20.3 or

if the substrate is a detectable fluorescent ester compound the MAF_C threshold value is calculated as an MDR activity factor for MDR1 + MRP1 and said threshold is equivalent with the one obtained for calcein.

14. The *in vitro* diagnostic method of any of claims 1 to 13 wherein the biological sample is blood sample and the patient is mammalian, preferably human.

15. Use of a kit for assessing the responsiveness of a sDMARD treated RA patient to bDMARD therapy before or at an initial phase of the bDMARD therapy, or for a purpose as defined herein,
by obtaining one or more transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient,
wherein
said RA patient is considered as a non-responder to the bDMARD therapy when the level of each transporter activity value is above a respective threshold level, or for a purposed, and
considering said RA patient as a responder to the bDMARD therapy when the level of each transporter activity value is not higher than a respective threshold level,
said kit comprising
- one or more substrate(s) of MDR1, MRP1 or both MDR1 and MRP1 for the measuring of the respective transporter activity,
said substrate being taken up by $CD3^+$ T-lymphocytes once contacted with them in a biological sample, wherein preferably said substrate is detectable, preferably fluorescent,
- optionally a label for $CD3^+$ T-lymphocytes,
and preferably
- inhibitor for MRP1 and/or,
- inhibitor for MDR1.

16. Use of a kit for assessing the responsiveness of a sDMARD according to claim 15,
for use in a method as defined herein or above or in a method according to of any of claims 1 to 14, wherein said kit comprises,

- a substrate for MDR1 and MRP1 for the measuring of a composite MDR1 and MRP1 transport activity, said substrate being taken up by leukocytes (preferably $CD3^+$ T-lymphocytes once contacted the reagents), in a biological sample,

wherein preferably said substrate is detectable, preferably fluorescent

or wherein said substrate transport is detectable, e.g. by ATP-ase activity

- label for $CD3^+$ T-lymphocytes,

and preferably

- inhibitor for MRP1,

- inhibitor for one or more other multidrug transporter.

17. Use of a kit according to any of claims 15 to 16 wherein the substrate is a calcein ester, preferably calcein AM, and

the activity is quantified as a multidrug activity factor (MAF),

and the inhibitors are selected from the group consisting of vinblastine, verapamil, vincristine, oligomycin, or cyclosporine.

18. A method for treating an RA patient by assessing the responsiveness of a sDMARD (synthetic disease-modifying antirheumatic drug) treated RA patient to bDMARD therapy (biological synthetic disease-modifying antirheumatic drug therapy), wherein preferably the patient is in need of a switch or modification of the sDMARD therapy, said method comprising the steps of

- providing a biological sample of said sDMARD treated RA patient, said sample comprising $CD3^+$ T-lymphocytes from said patient,

- obtaining one or more transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD treated RA patient, before or at an initial phase of a bDMARD therapy, by using one or more substrate(s) of MDR1, MRP1 or of both MDR1 and MRP1,

- comparing the one or more transporter activity value(s) with one or more pre-determined threshold transporter activity level(s),

wherein each pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters and which has been determined using the same one or more substrates,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of said one or more transporter activity value(s) measured is above said threshold level, and

- considering said RA patient as a responder to the bDMARD therapy when the level of each transporter activity value is not higher than said threshold level,

- wherein the RA patient is a responder to the bDMARD therapy, carrying out a bDMARD therapy,

- wherein the RA patient is a non-responder to the bDMARD therapy, carrying out an alternative therapy,

wherein preferably said bDMARD therapy is selected from the group consisting of

- anti-TNF therapy,
- T-cell activation inhibitor therapy,
- B lymphocyte depletion therapy,
- anti-IL6 therapy, preferably
- anti-TNF therapy and
- T-cell activation inhibitor therapy
- wherein preferably the sDMARD therapy is a preferably csDMARD therapy.

19. The method of claim 18, wherein

the transporter activity value is obtained by measuring transport activity of one or more multidrug transporters comprising at least MDR1 in the $CD3^+$ T-lymphocytes of said sDMARD treated RA patient before a bDMARD therapy or in an initial phase thereof, by using an MDR1 substrate, and

the MDR1 activity value is compared with a pre-determined threshold transporter activity level, wherein said pre-determined threshold transporter activity level has been determined using the same MDR1 substrate,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of the MDR1 activity value is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of the MDR1 activity value is not higher than said threshold level
- wherein the RA patient is a responder to the bDMARD therapy, carrying out a bDMARD therapy,
- wherein the RA patient is a non-responder to the bDMARD therapy, carrying out an alternative therapy.

20. The method of claim 19, wherein

the transporter activity value is obtained by measuring transport activity of one or more multidrug transporters comprising MDR1 and MRP1, in the $CD3^+$ T-lymphocytes of said sDMARD treated RA patient before a bDMARD therapy or in an initial phase thereof, and

the transporter activity value is compared with a pre-determined threshold transporter activity level, wherein said pre-determined threshold transporter activity level has been determined using the same substrate,

- considering said RA patient as a non-responder to the bDMARD therapy when the level of the MDR1 and MRP1 transporter activity value is above said threshold level, and
- considering said RA patient as a responder to the bDMARD therapy when the level of the MDR1 and MRP1 transporter activity value is not higher than said threshold level,
- wherein the RA patient is a responder to the bDMARD therapy, carrying out a bDMARD therapy,
- wherein the RA patient is a non-responder to the bDMARD therapy, carrying out an alternative therapy.

21. The method of claim 20 wherein

wherein said MDR1 and MRP1 activity is measured with a substrate of both MDR1 and MRP1, the transporter activity comprising activities of both MDR1 and MRP1 and the two transporters are not differentiated by inhibition, whereby a composite transporter MDR1-MRP1 activity value is obtained.

22. The method of any of claims 18 to 21 wherein said threshold transporter activity level has been determined by using the one or more substrates by

measuring or quantifying the transport activity of said one or more multidrug transporters in the $CD3^+$ T-lymphocytes in a reference patient group known to be responder to the bDMARD therapy and a further reference patient group known to be non-responder to the bDMARD therapy, and

the transport activity values measured in the responder and non- responder patient groups are statistically analysed as distributions to find a threshold level which differentiates between responder transport activity values and non-responder transport activity values.

23. The method of any of claims 18 to 22 wherein measuring the transporter activity comprises

- contacting at least the $CD3^+$ T-lymphocytes in the biological sample with the one or more transporter substrate(s), said substrate being a derivative of a detectable fluorescent compound, and wherein said derivative is taken up by at least the $CD3^+$ T-lymphocytes and is hydrolyzed into said fluorescent compound in the cells, wherein said fluorescent compound is not transportable by MDR1 or MRP1 or by neither MDR1 nor MRP1 or transportable to a significantly lesser extent than the ester derivative, and
- measuring fluorescence in the $CD3^+$ T-lymphocytes,
- obtaining the transport activity value from the fluorescence in the $CD3^+$ T-lymphocytes,

wherein preferably

at least MDR1 activity is measured and/or

at least a composite transporter activity is measured, with a substrate of both MDR1 and MRP1, wherein the two transporters are also not differentiated by inhibition and a composite activity value is obtained.

24. The method of any of claims 18 to 23 wherein the substrate is a detectable fluorescent ester compound.

25. The method of claim 24 wherein

measuring transport activity is based on the difference between the fluorescence of the detectable fluorescent compound in the cells measured in the presence of an inhibitor of the multidrug transporters and the fluorescence measured in absence of said inhibitor,

wherein preferably measurement is carried out by flow cytometry.

26. The method of any of claims 24 to 25 wherein the biological sample is peripheral blood and the substrate is calcein ester and the transporter activity (value) threshold level is calculated as an MDR activity factor for MDR1 (preferably MAF_{mdr1}) and said threshold is between 15 to 19, preferably 16 to 19 or 16 to 18, more preferably between 17 to 19 or 17 to 18, preferably about 17.4, or
the substrate is a detectable fluorescent ester compound the transporter activity (value) threshold level is calculated as an MDR activity factor for MDR1 (preferably MAF_{MDRI}) and said threshold is equivalent with the one obtained for calcein.

27. The method of any of claims 24 to 26 wherein the biological sample is peripheral blood and the MDR1 substrate is calcein ester and the transporter activity (value) threshold level is calculated as a composite MDR activity factor for MDR1 and MRP1 (preferably MAF_c) and said threshold is between 19 to 23, preferably 20 to 22 more preferably between 20.8 to 21.8, preferably about 21.3, or
the substrate is a detectable fluorescent ester compound the transporter activity (value) threshold level is calculated as an MDR activity factor for MDR1 (preferably MAF_{MDRI}) and said threshold is equivalent with the one obtained for calcein.

28. The method of any of claims 18 to 27 wherein the initial phase of the bDMARD therapy is no longer than 8 weeks, preferably 7 weeks or 6 weeks, highly preferably 4 weeks, even more preferably 2 weeks.

29. The method of any of claims 18 to 28, wherein said sDMARD-treated RA patient has been also treated by bDMARD therapy,
said method additionally comprising
- providing a further biological sample of said sDMARD-treated RA patient between weeks 4 and 7 of the bDMARD therapy,
- obtaining one or more further transporter activity value(s) by measuring transport activity by one or more multidrug transporter(s) selected from the group consisting of MDR1 and MRP1 in the $CD3^+$ T-lymphocytes of said sDMARD (preferably csDMARD) treated RA patient between weeks 4 and 7 of the bDMARD therapy, by using one or more substrate(s) of MDR1, MRP1 or both MDR1 and MRP1,
- comparing one or more further transporter activity value(s) with one or more pre-determined threshold transporter activity level(s),
wherein each pre-determined threshold transporter activity level is a threshold value for the transport activity of said one or more multidrug transporters and which has been determined using the same one or more substrates, as defined above,
- considering said RA patient as a non-responder to the biological therapy wherein the level of each transporter activity value measured is above said each threshold level, and
- considering said RA patient as a responder to the biological therapy wherein the level of each transporter activity value is not higher than said each threshold level

wherein preferably the bDMARD therapy is a bDMARD therapy as defined in claim 4, and
wherein preferably measuring or quantifying transporter activity of transporter protein is carried out as defined in claims 1 to 10, preferably in claim 6 or in claims 9 or 10, and
wherein preferably the substrate is a substrate of both MDR1 and MRP1 and highly preferably the substrate is a calcein ester.

30. The method of claim 29 wherein
- at least the MDR1 activity is measured, and the transporter activity value is $MAF_{MDR}I$ value, and said RA patient is considered as a non-responder to biological therapy wherein if the substrate is a calcein ester then the level of $MAF_{MDR}I$-is above a $MAF_{MDR}I$ threshold from 12 to 15, preferably above 13.9 or
  if the substrate is a detectable fluorescent ester compound the $MAF_{MDR}I$ threshold value is calculated as an MDR activity factor for MDR1 (preferably $MAF_{MDR}I$) and said threshold is equivalent with the one obtained for calcein;
- at least the MRP1 activity is measured, and the transporter activity value is $MAF_{MRP}I$ value, and said RA patient is considered as a non-responder to biological therapy wherein the level of $MAF_{MDRP}I$-is above a $MAF_{MRP}I$ threshold from 5 to 7, preferably above 6.0 or
  if the substrate is a detectable fluorescent ester compound the $MAF_{MRP}I$ threshold value is calculated as an MDR activity factor for MRP1 (preferably $MAF_{MRP}I$) and said threshold is equivalent with the one obtained for calcein
- at least a composite activity is measured, and the transporter activity value is MAF_C value, and said RA patient is considered as a non-responder to biological therapy wherein the level of MAF_c.is above a MAF_C threshold from 18 to 22, preferably above 20.3 or
  if the substrate is a detectable fluorescent ester compound the MAF_C threshold value is calculated as an MDR activity factor for MDR1 + MRP1 and said threshold is equivalent with the one obtained for calcein.

31. The method of any of claims 18 to 30 wherein the biological sample is blood sample and the patient is mammalian, preferably human.

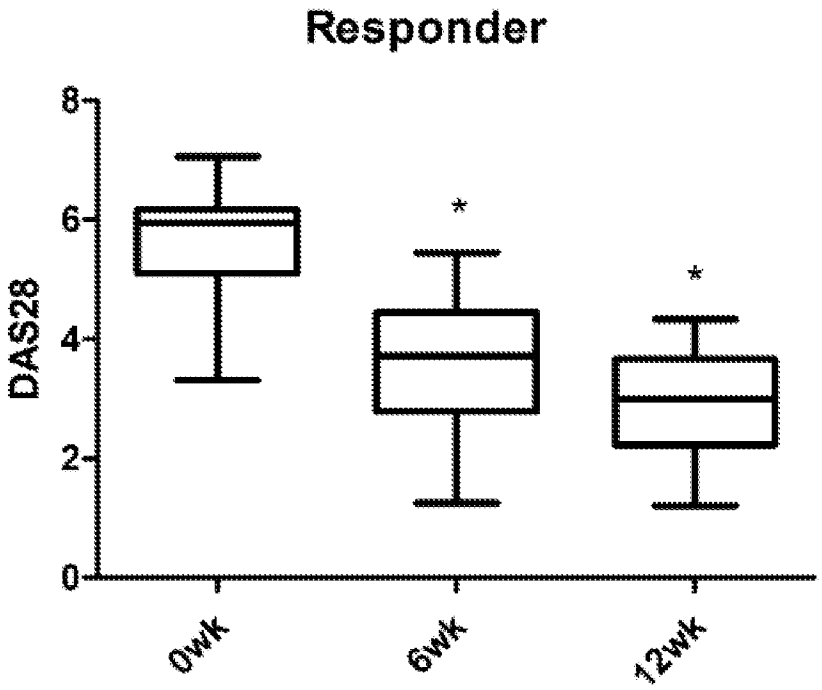

FIGURE 1.A

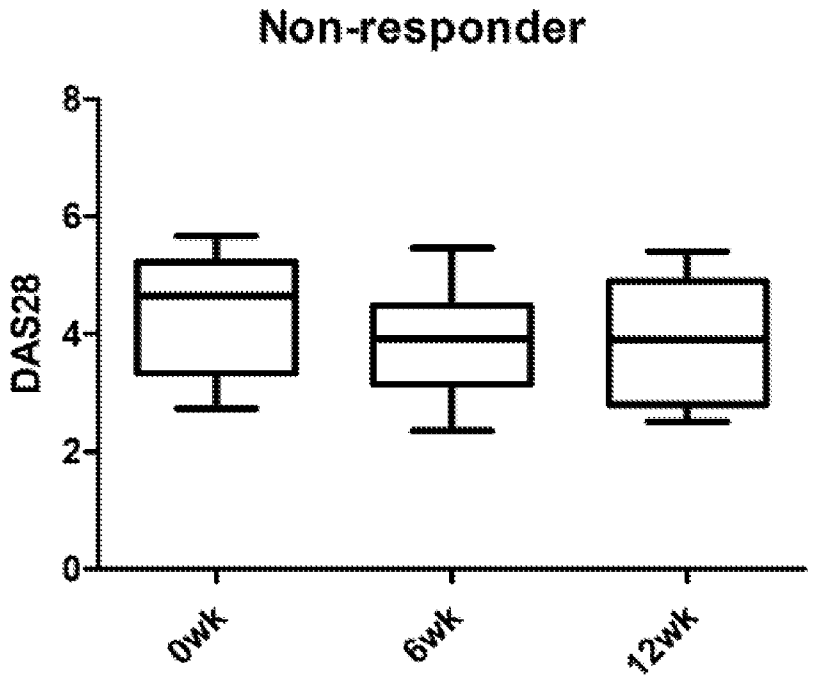

FIGURE 1.B

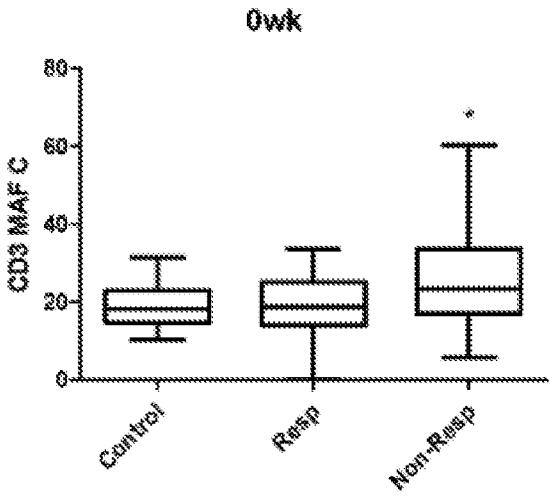

FIGURE 2.A.1

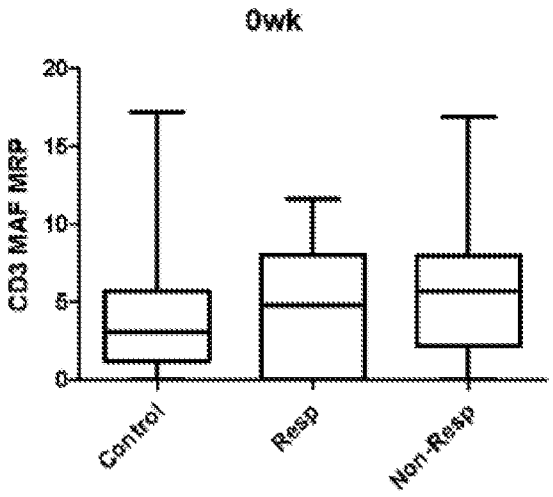

FIGURE 2.A.2

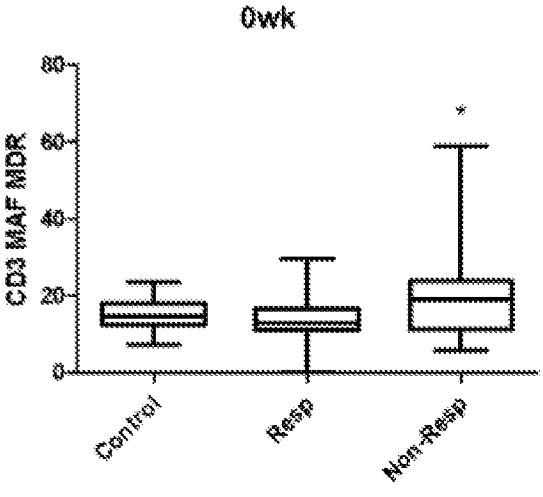

FIGURE 2.A.3

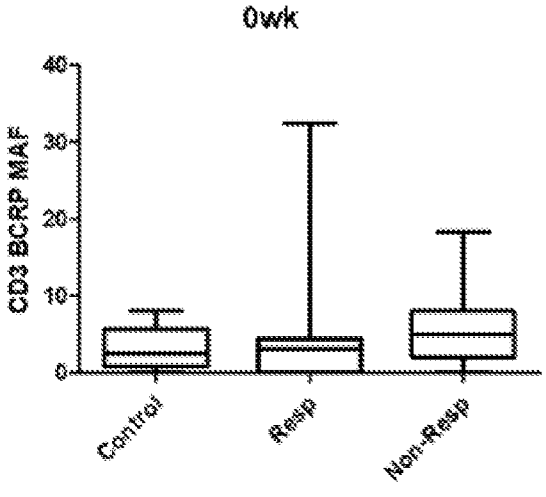

FIGURE 2.A.4

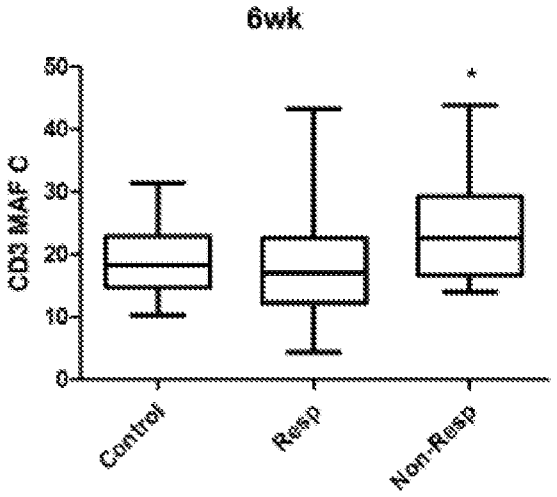

FIGURE 2.B.1

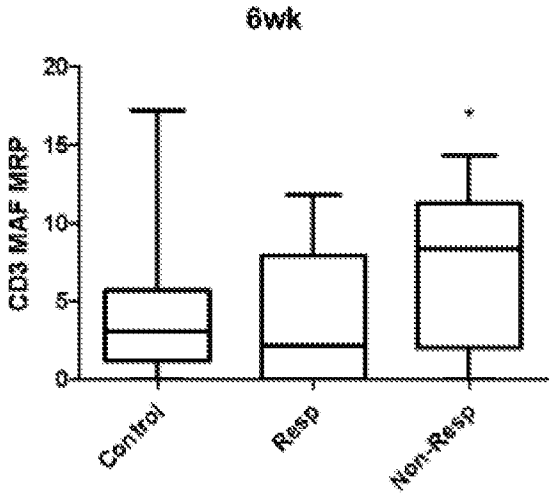

FIGURE 2.B.2

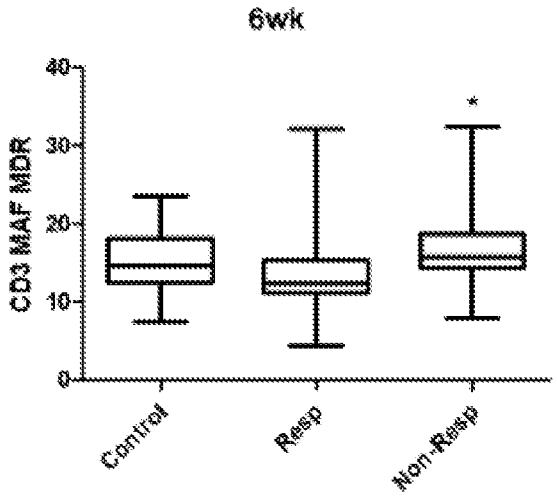

FIGURE 2.B.3

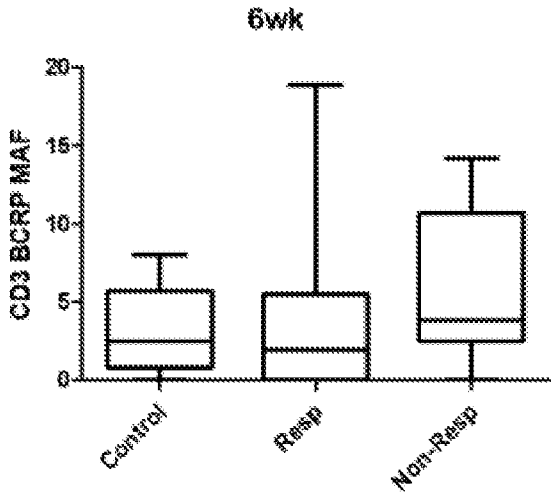

FIGURE 2.A.4

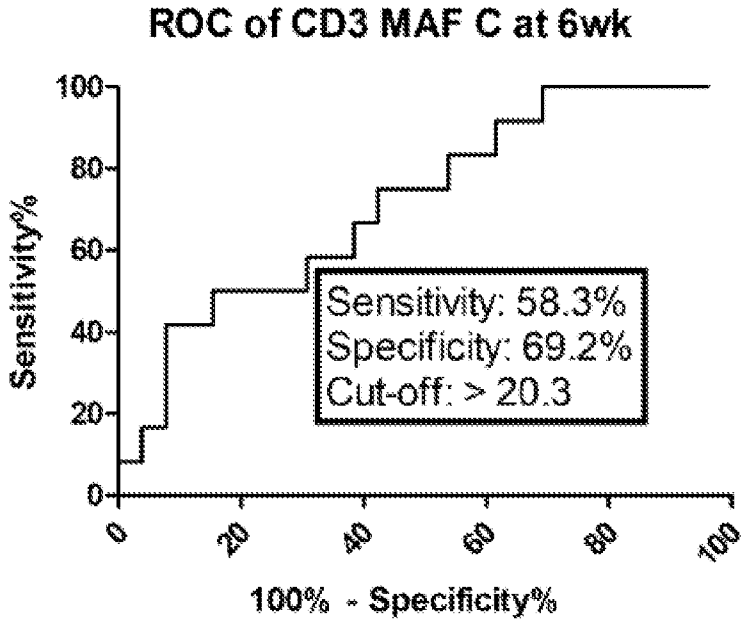

FIGURE 3.A

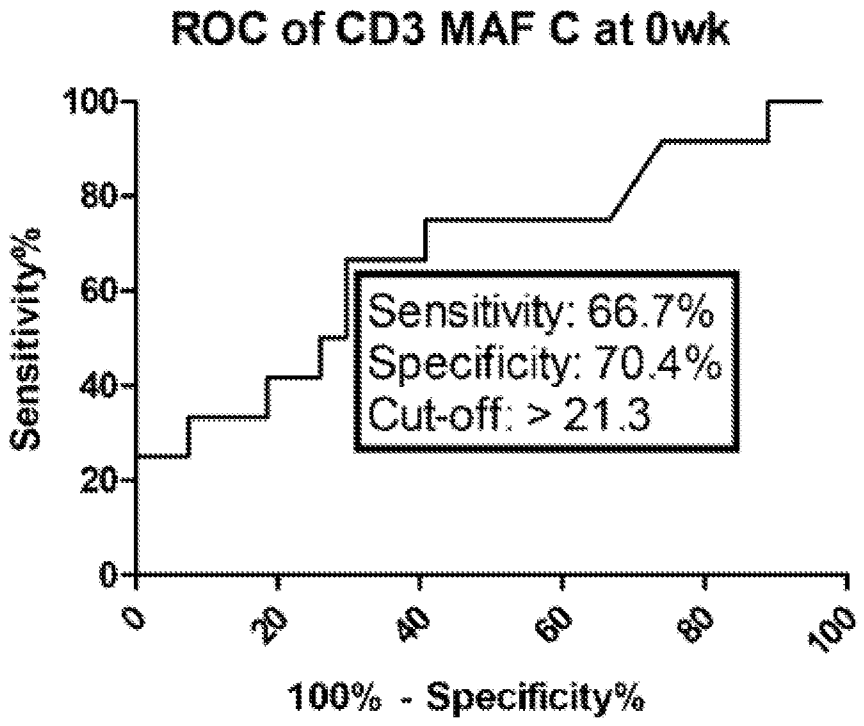

FIGURE 3.B

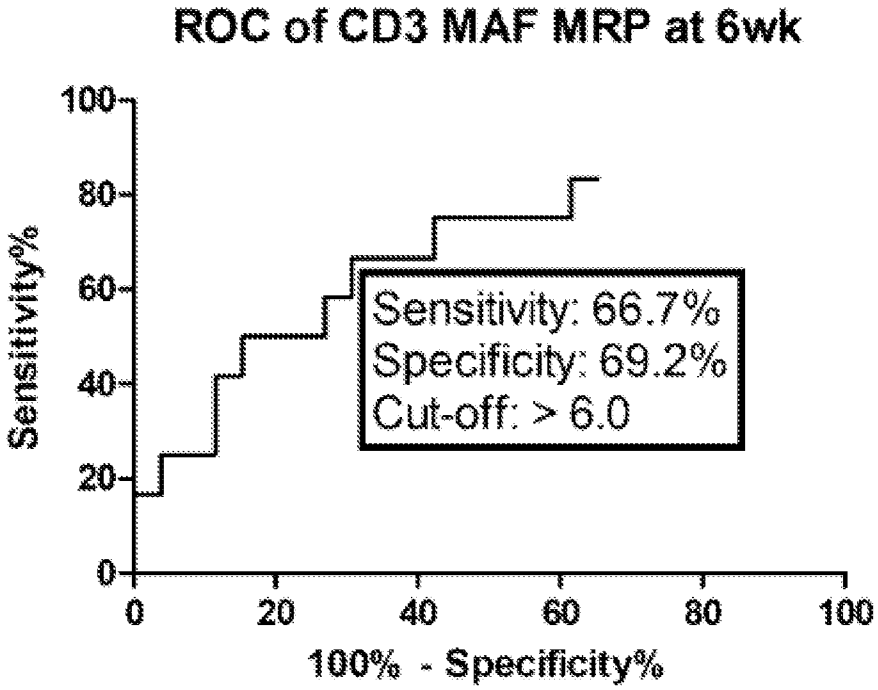

FIGURE 3.C

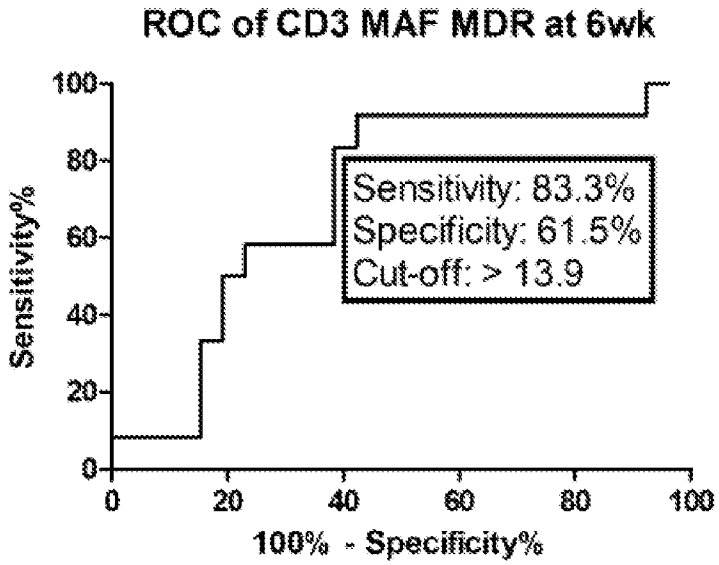

FIGURE 3.D

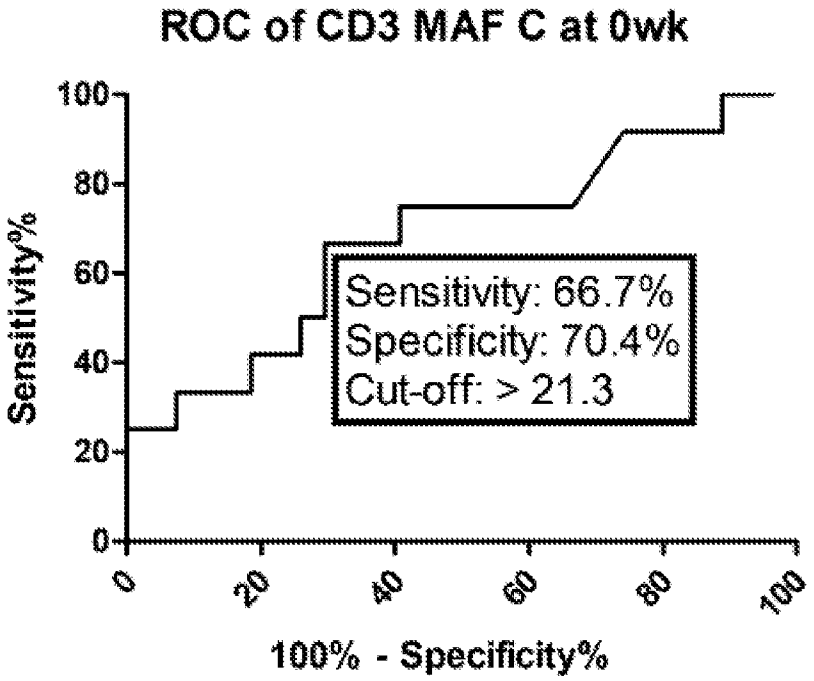

FIGURE 4.A

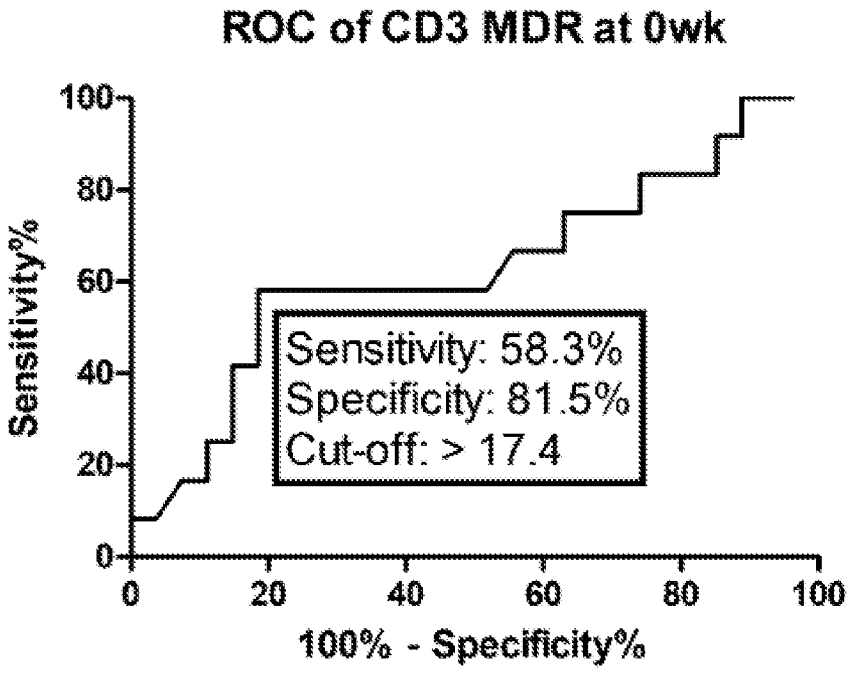

FIGURE 4.B

INTERNATIONAL SEARCH REPORT

International application No
PCT/HU20 19/050025

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 G01N33/564
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SZEKANECZ Z ET AL: "OP0230 MULTI DRUG RESISTANCE (MDR) PROTEIN ACTIVITY OF ACTIVATED T LYMPHOCYTES IS A PREDICTOR OF BIOLOGICAL TREATMENT RESPONSE IN RHEUMATOID ARTHRITIS", ANNALS OF THE RHEUMATIC DISEASES, BRITISH MEDICAL ASSOCIATION, GB, vol. 75, no. Suppl. 2, 31 May 2016 (2016-05-31), page 145, XP009507948, ISSN: 0003-4967, DOI: 10.1136/ANNRHEUMDIS-2016-EULAR.3369 title; abstract ----- -/--	1-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 October 2019	31/10/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hohwy, Morten

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IRINA V. LEBEDEVA ET AL: "Sensitive and Specific Fluorescent Probes for Functional Analysis of the Three Major Types of Mammalian ABC Transporters", PLOS ONE, vol. 6, no. 7, 22 July 2011 (2011-07-22), page e22429, XP055506228, DOI: 10.1371/journal.pone.0022429 abstract; fig. 4	1-31
Y	TAM?S MICSIK ET AL: "Decreased functional activity of multidrug resistance protein in primary colorectal cancer", DIAGNOSTIC PATHOLOGY, BIOMED CENTRAL LTD, LO, vol. 10, no. 1, 16 April 2015 (2015-04-16) , page 26, XP021215217, ISSN: 1746-1596, DOI: 10.1186/S13000-015-0264-6 abstract; p.2-3; p. 3, col. 2, par. 3	1-31