Original Article

Determination of reference values of MDR-ABC transporter activities in CD3+ lymphocytes of healthy volunteers using a flow cytometry based method

Péter Szerémy,[1,2] Katalin Tauberné Jakab,[2] Sándor Baráth,[3] András Apjok,[1,2] Kata Filkor,[1] Zsolt Holló,[2] János Márki-Zay,[2] János Kappelmayer,[3] Sándor Sipka,[4] Péter Krajcsi,[2] and Gergely Toldi[1]*

[1]MDQuest Ltd, Szeged, Hungary
[2]SOLVO Biotechnology, Budaörs, Hungary
[3]Department of Laboratory Medicine, University of Debrecen, Debrecen, Hungary
[4]3rd Department of Internal Medicine, University of Debrecen, Debrecen, Hungary

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

Correspondence to: Gergely Toldi MD PhD MDQuest Ltd, Közép fasor 52, Szeged, H-6726, Hungary. Email: toldi@mdquest.hu

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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

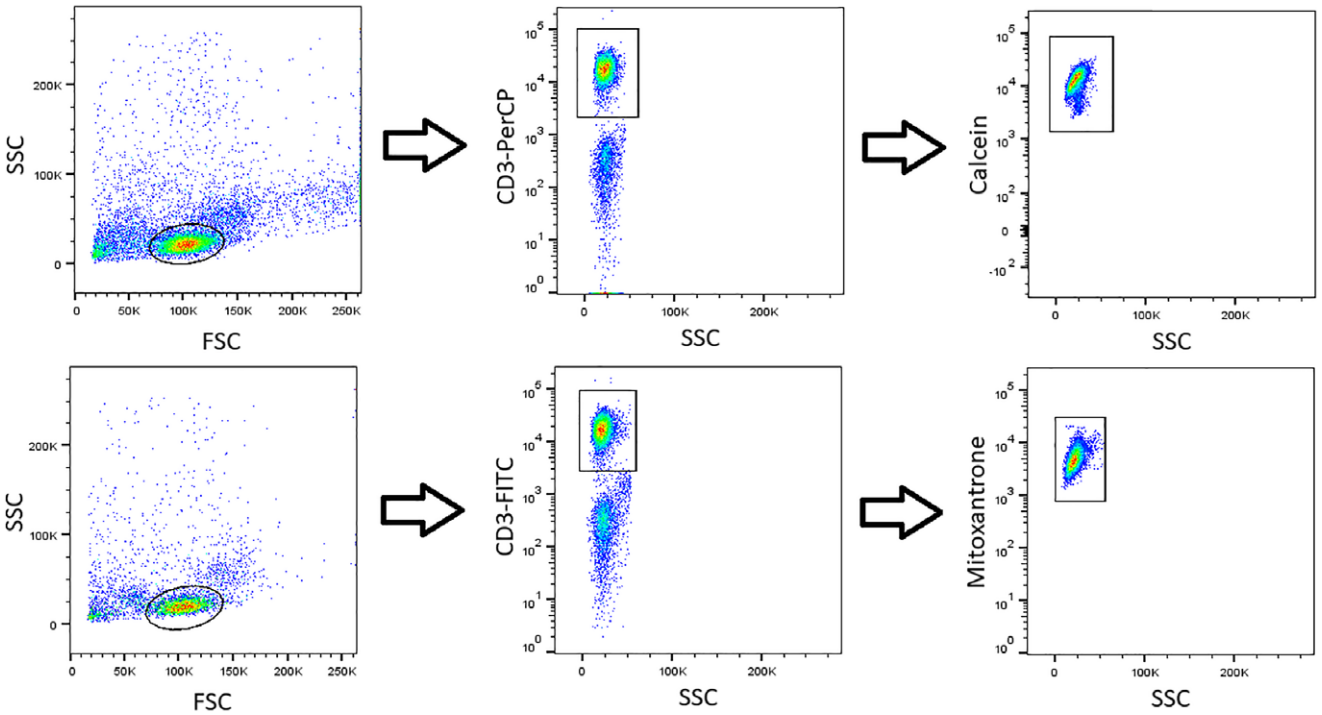

FIG. 1. Gating strategy to determine Calcein and mitoxantrone fluorescence on a representative sample. FSC, forward scatter characteristics; SSC, side scatter characteristics.

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

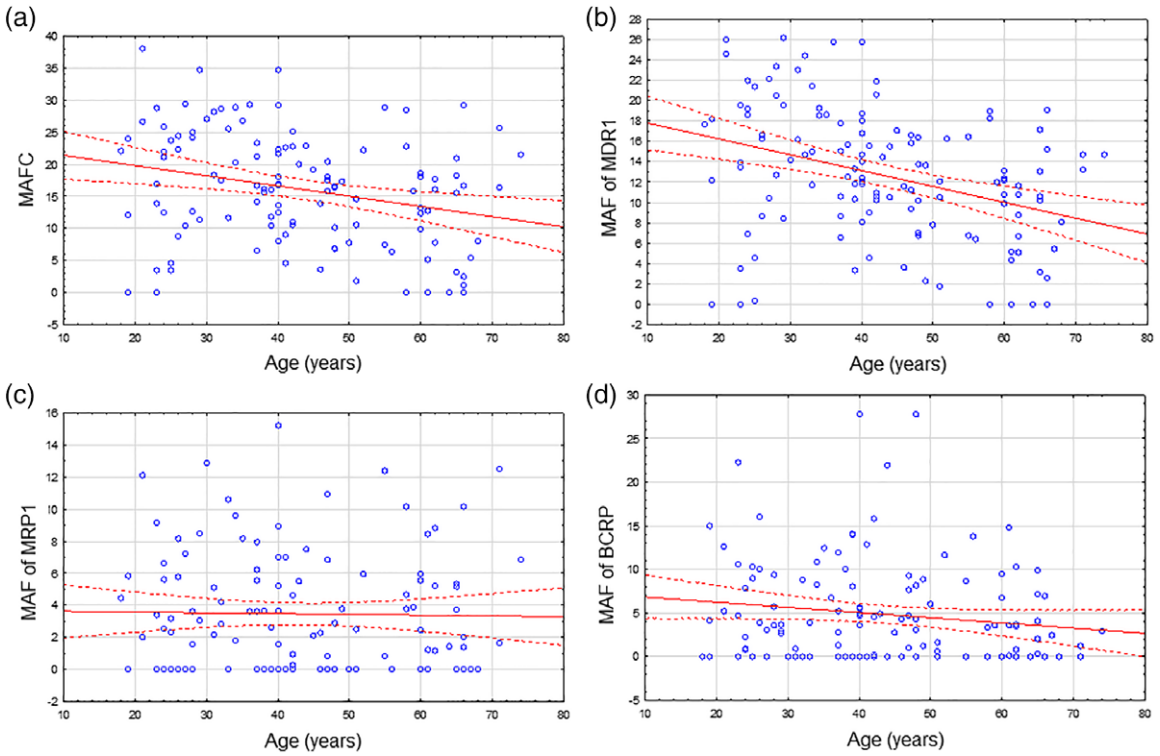

FIG. 2. Scatterplots of the MAFC value (a) and MAF values of MDR1 (b), MRP1 (c) and BCRP (d) against age. 95% confidence interval is represented in red. MAFC and MAF of MDR1 show negative correlation with the age of the studied subjects.

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.

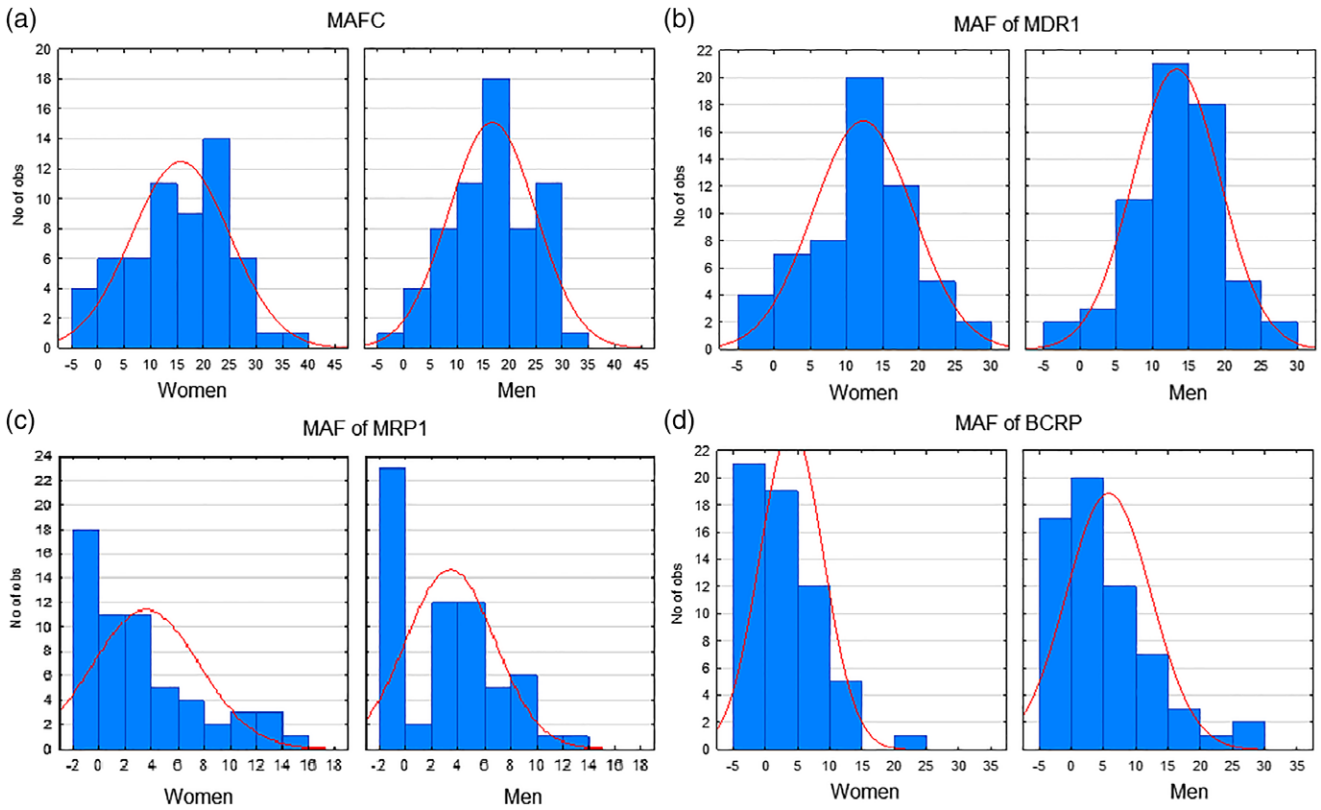

FIG. 3. Histograms representing the distribution of the MAFC value (a) and MAF values of MDR1 (b), MRP1 (c), and BCRP (d) in men and women. Gender does not affect the presence or lack of correlation between MAF values and age.

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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