

LYMPHOCYTE P-GLYCOPROTEIN VARIABILITY IN HEALTHY INDIVIDUALS

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Abstract The aim of the present work was to describe the distribution of lymphocyte P-glycoprotein activity on a population of healthy individuals, taking also into account sex and age. P-glycoprotein activity in lymphocytes was measured by the Rhodamine 123 efflux assay using flow cytometry, in the presence and absence of verapamil, a P-glycoprotein inhibitor. We obtained a range of P-glycoprotein activity from 1.04 to 3.79. The distribution of the activity in the population studied was better described by a bimodal model, according with the Kolmogorov-Smirnov test. The frequency adjusted to the following equation: $F = 0.70 N(2.11; 0.43) + 0.30 N(3.29; 0.26)$, in which 0.70 and 0.30 represented the proportion of each group, and 0.43 and 0.26 were the standard deviations of the activity of each group, respectively. The study of the relationship between subjects' age and P-glycoprotein activity showed no statistical significance. When healthy volunteers were separated according to sex, similar distributions were observed, although for men an increase in proportion of higher P-glycoprotein function group was observed. The variability observed in the population studied was important, with some volunteers with very scarce activity and some with a fourfold higher activity. Characterization of P-glycoprotein functionality in the population represents a useful contribution to the beginning of pharmacological treatments that consider its effect on pharmacokinetics and pharmacodynamics of individualized patients.

Key words: P-glycoprotein, ABCB1, Rh123 efflux assay, drug transporters

Resumen *Variabilidad de la glicoproteína P linfocitaria en una población sana.* El objetivo del presente trabajo fue describir la distribución de la actividad de la glicoproteína P linfocitaria en una población de individuos sanos, considerando a su vez el sexo y la edad. La funcionalidad de la glicoproteína P fue determinada mediante el ensayo de eflujo de Rodamina 123, en presencia y ausencia de verapamilo, un inhibidor competitivo de este transportador, determinando la fluorescencia intracelular remanente mediante citometría de flujo. Obtuvimos un rango de actividades de entre 1.04 y 3.79. La distribución de la actividad en la población evaluada se ajusta a un modelo bimodal, según el test de Kolmogorov-Smirnov. La frecuencia ajusta a la siguiente ecuación: $F = 0.70 N(2.11; 0.43) + 0.30 N(3.29; 0.26)$ donde 0.70 y 0.30 representan las proporciones de cada grupo, mientras que 0.43 y 0.26 corresponden al desvío estándar de la actividad de cada grupo respectivamente. Al estudiar la correlación entre la edad de los sujetos y la función de la proteína, no se observaron diferencias significativas. Cuando los individuos fueron clasificados en función del sexo, las distribuciones obtenidas fueron semejantes, aunque para los varones se observó un aumento en la proporción de individuos con alta actividad. La variabilidad observada fue importante, comprendiendo individuos con escasa actividad y otros que presentaron una actividad hasta cuatro veces mayor. La caracterización de la función de la glicoproteína P en la población representa una contribución indispensable para el desarrollo de tratamientos farmacológicos personalizados que consideren el efecto de dicho transportador en la farmacocinética y farmacodinámica de cada paciente.

Palabras clave: glicoproteína P, ABCB1, ensayo de eflujo de Rh123, transportadores de fármacos

Within pharmacological development, great population variability has been detected in drug absorption, distribution, metabolism, elimination and toxicity (ADMET). Partly, these variations have been explained by individual me-

tabolizing capability determined by cytochrome P450 family. Nevertheless, the impact of these enzymes cannot completely justify the individual variability observed in pharmacokinetics and pharmacodynamics¹.

Metabolizing enzymes are now known to be articulated with membrane efflux transporters as a protection mechanism of the cells in which toxic and harmful compounds coming from the environment are transformed and extruded by these proteins². Between membrane transporters substrates, a huge number of clinical drugs have been found. In mammals, the ATP-binding cassette (ABC)

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superfamily was first described as responsible for the multidrug resistance phenotype. Nowadays, these biological pumps have been identified as having a major impact on the pharmacological behavior of most drugs in use².

ABC transporters are present in all cells of all organisms and use the energy of ATP hydrolysis to transport substrates across cell membranes³. The first member described, and by now the most studied, was P glycoprotein (P-gp), codified in the ABCB1 gene, which received its name due to its effect in reducing plasmatic membrane drug permeability⁴.

The most striking property of P-gp is its ability to transport an incredibly diverse range of compounds which do not share obvious structural characteristics⁵, other than their amphipatic nature. It has been recently suggested that this multispecificity might be consequence of a single, large, flexible hydrophobic pocket in which drugs are essentially shielded from both lipid and aqueous phases⁵. P-gp has been involved in relevant clinical drug transport such as antibiotics, antimalarials, analgesics, antiretrovirals, chemotherapeutic drugs, and also fluorescent dyes.

This efflux pump has a ubiquitous expression in tissues including intestine, liver, kidney, blood brain barrier, placenta and cells from the hematological compartment. P-gp must have evolved, in part, to protect the brain from damaging effects of xenotoxins that can be incorporated through food or generated by pathogenic organisms in the intestine². This statement comes from the observation that double knock-out mice have shown to be fertile and present no abnormalities, other than an augmented neurotoxic effect of drugs⁶. The bioavailability of drugs classified as P-gp substrates is increased in these animals⁷.

Additionally, Chaudhary et al. have documented that P-gp is expressed and active in human peripheral blood lymphocytes⁸. Physiological function of P-gp in the hematological compartment has been partially described as transporter of lymphokines and growth factors related with differentiation and maturation. In pathological conditions, like acute myeloid leukemia, this pump has been associated with resistance to therapeutics and bad prognosis of the disease⁹.

The role of P-gp in human physiology and pharmacology has been extensively reviewed^{10, 11}. Inter-individual variability in P-gp function becomes relevant considering that it determines drug absorption from the gastrointestinal tract, drug distribution into the CNS and drug elimination via bile, direct intestinal secretion and urine of numerous clinically important compounds¹².

The aim of the present work was to describe the distribution of lymphocyte P-gp activity on a population of healthy individuals, taking into account sex and age.

Materials and Methods

Sixty-one healthy volunteers from Buenos Aires city were included in this protocol. The subjects were ascertained to be healthy by a questionnaire and routine laboratory tests. Information requested was age, sex, race, height, weight, nationality, smoking and nutritional habits, consume of alcohol and drugs. Biochemical determinations performed were hemogram, erythro sedimentation, glycemia, creatininemia, total cholesterol, triglycerides, total bilirubin and transaminases. The age range was from 19-90 years old, 38 ± 2 years (mean \pm standard deviation).

Samples were collected after informed consent was signed by the donors. This study was approved by the Clinical Investigation Ethics Committee (CETIN).

Venous blood samples were collected with heparin in sterile condition. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as previously described¹³. Briefly, blood was diluted 1:3 with physiological solution and centrifuged for 20 min at 2000 r.p.m. in Ficoll-Paque. The monolayer was carefully isolated. Then washed twice with phosphate buffer salt (PBS) and resuspended in RPMI 1640.

Cell viability was determined by trypan blue, requiring more than 90% of viability to perform the assay.

P-gp activity was determined by Rhodamine 123 (Rh123) efflux assay¹⁴. 10^6 cells were exposed to 150 ng ml^{-1} ($0.39 \mu\text{M}$) of Rh123 for 10 min at room temperature in the dark. After two washes with cold PBS, cells were resuspended in RPMI 1640-Rh123 free and 10% fetal bovine serum (FBS), with and without Verapamil ($100 \mu\text{M}$), and incubated for 3 h at 37°C in a 5% CO_2 atmosphere. Cells were then washed and resuspended with PBS and kept on ice until analysis.

To verify the effect of the protein on the transport, negative control of activity was simultaneously evaluated with 3 h incubation at 4°C .

Fluorescence measurements (Rho123: 505 nm excitation wavelength; 534 nm emission wavelength) of individual cells were performed with a Becton-Dickinson Fluorescence Activated Cell Sorter (FACS) Calibur cytometer equipped with an argon laser. Data was acquired from 10 000 cells. Lymphoid region was selected, based on size and granularity (Fig. 1), and log fluorescence was collected and displayed as a single parameter histogram. Geometric mean fluorescence intensity (because the data is acquired as a logarithm) was obtained. P-gp activity was calculated as the ratio of mean fluorescence intensity for Rh123 in Rh123 + Verapamil/Rh123 as recommended by Marie¹⁵.

The coefficient of interassay variation was determined by repeating the method five times with a sample taken from a unique donor obtaining a value of 13%.

RPMI 1640, FBS and PBS were acquired from Gibco. Ficoll-Paque PLUS was provided by Amersham Biosciences. Rhodamine 123 and Verapamil hydrochloride were dissolved in distilled water, fractioned and kept at -20°C until use (Sigma-Aldrich, USA). PSC833 was generously provided by Novartis, Switzerland.

The normal and bimodal distribution of P-gp activity in the population as well as sex differences were analyzed by the Kolmogorov-Smirnov test¹⁶. Age and P-gp activity were correlated by linear regression analysis.

The differences between men and women were estimated by Mann-Whitney U-two tailed-test¹⁷. The difference in proportion of men and women groups were estimated by a test for proportions¹⁸.

In all cases, *p*-values < 0.05 were considered significant.

Results

The samples evaluated were from Caucasian healthy adults. Demographic characteristics are resumed in Table 1.

In order to study lymphocytic P-gp functionality, an efflux assay was carried out. Cells were preloaded with the probe of interest (Rh123), washed twice with PBS and the resulting intracellular fluorescence was measured by FACS flow cytometry, after incubation with and without calcium blocker verapamil, a P-gp competitive inhibitor. Inhibition of the transporter would result in a major amount of probe in the cell¹⁹. Rh123 efflux index value was determined by the ratio of fluorescence between inhibition and activity. As lymphocytes are identified by physical parameters (Fig. 1), the fluorescence obtained in each condition is generated by the remaining probe in each cell, according to the level of P-gp activity which varies depending on the cell lineage²⁰. Therefore, our results refer to the mean of P-gp activity considering all the lymphoid lineages present in peripheral blood.

Figure 2 shows that chemical competitive inhibition with Verapamil (100 µM) as well as physical inhibition at 4 °C (lowering metabolism) showed less Rh123 efflux compared with control cell incubated at 37 °C. Similar results were obtained with PSC833 (1 µM) as inhibitor.

In the population studied, we observed that Rh123 efflux assay yielded a great dispersion in P-gp activity values ranging from 1.04 to 3.79 (Fig. 3). The distribution of P-gp activity was better described by a bimodal model, according to Kolmogorov-Sirnov test. The frequency adjusts to the following equation:

$$F = 0.70 N (2.11; 0.43) + 0.30 N (3.29; 0.26)$$

where 0.70 and 0.30 represent the proportion of each group and 0.43 and 0.26 the standard deviations of P-gp activity of each group, respectively. P-gp activity differences between these two groups are statistically significant (*p* < 0.05).

TABLE 1.– Demographic table of subjects studied

	Women	Men	Population
Number of donors	36	25	Total: 61
Height (cm) $\bar{X} \pm SD$	162 ± 6	175 ± 7	167 ± 9
Weight (kg) $\bar{X} \pm SD$	60 ± 12	75 ± 9	66 ± 13

Taking this in account, a ratio around 2.1 represents a low P-gp activity, while values around 3.3 may represent a major P-gp activity, revealed by a higher efflux of the dye.

The study of the relationship between subjects' age and P-gp activity showed no statistical significance (Fig. 4).

When volunteers were grouped according to sex, similar bimodal distributions were observed. In this case, the equations were:

$$F = 0.82 N (2.09; 0.48) + 0.18 N (3.37; 0.12) \text{ Women}$$

$$F = 0.56 N (2.18; 0.35) + 0.44 N (3.25; 0.31) \text{ Men}$$

According to the equations above, for women there was a proportion of 0.82 with low activity and 0.18 with high activity. Meanwhile, for men, a proportion of 0.56 presented low activity and 0.44 high. P-gp activity mean was similar in women and men (Figure 5). Although for men an increase in proportion of higher P-gp activity group was observed (*p* < 0.05).

Discussion

In our work, we investigated P-gp activity in lymphocytes of healthy volunteers.

The variability observed in the population studied was important, with some volunteers with very scarce activity and some with a fourfold higher activity. The analysis of

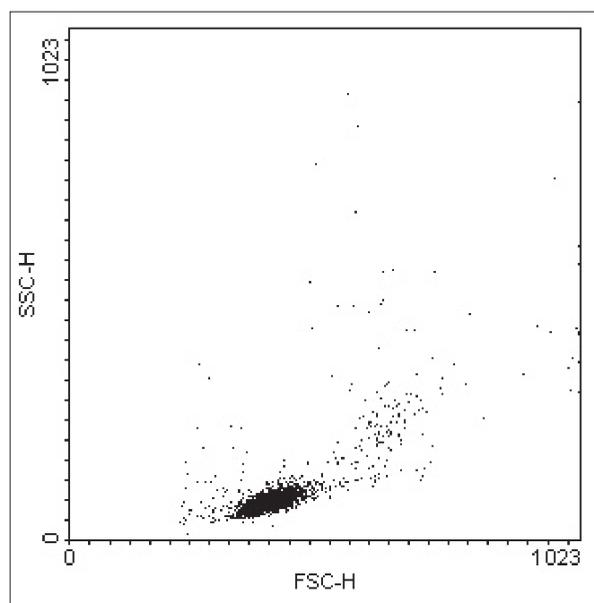


Fig. 1.– Dot-plot from mononuclear cells isolated from whole blood. Data obtained from side scattered (SSC) and forward scattered (FSC-H) light in a flow cytometer, defining cellular size and granularity.

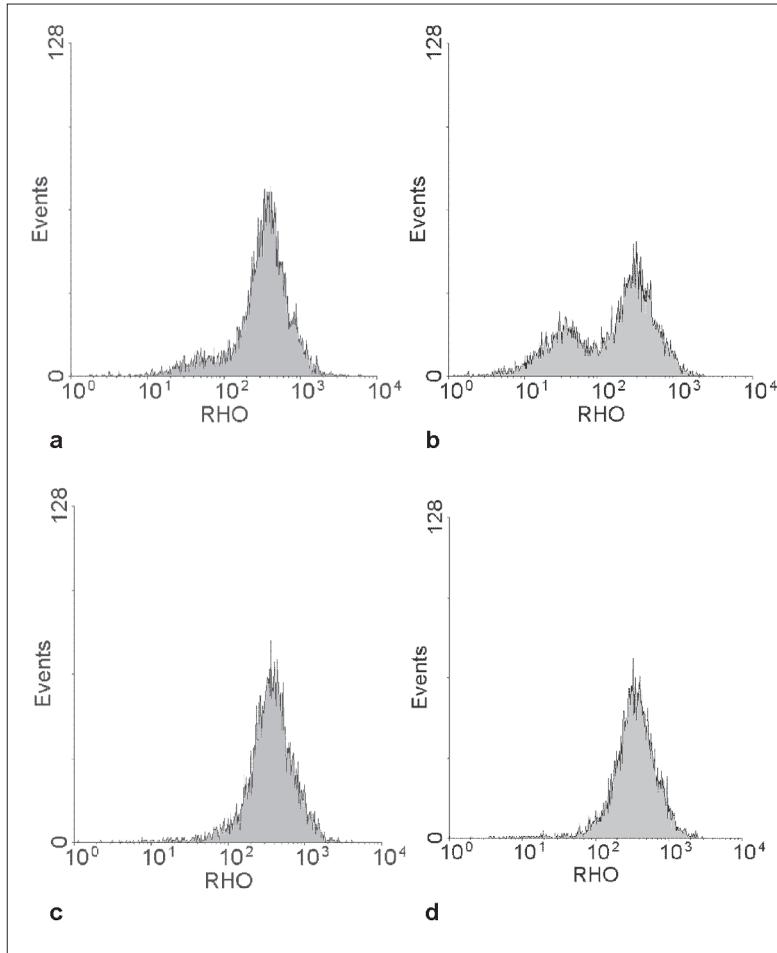


Fig. 2.— Rhodamine 123 fluorescence in lymphocytes. Logarithmic fluorescence intensity in lymphocytes stained with Rhodamine 123 (RHO) and incubated for 3 h in dye free media at 4 °C (a) and 37 °C (b); at 4 °C (c) and 37 °C (d) in the presence of verapamil (100 µM). a. Control at 4°C. b. Control at 37°C. c. Inhibitor at 4 °C. d. Inhibitor at 37 °C

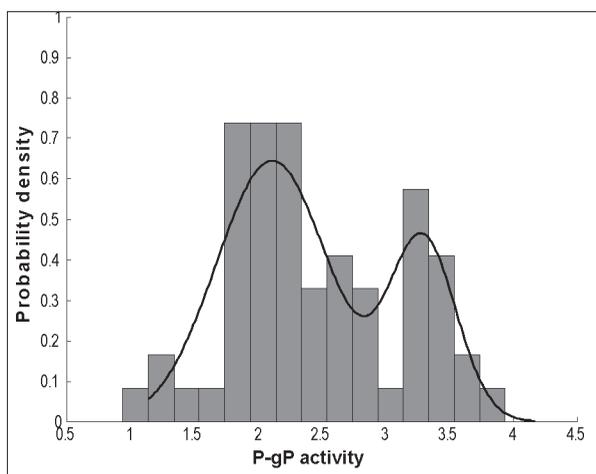


Fig. 3.— Distribution of P-gp activity among 61 healthy Caucasian subjects. P-gp is expressed as the ratio of mean fluorescence intensity for Rh123 as Rh123 + verapamil/ Rh123.

P-gp activity distribution showed a bimodal model, and no relationship between the subjects' age or sex and P-gp activity was found.

The lack of differences in P-gp activity between sex was previously described in the intestine²¹. Although we made the same observation in lymphocytes comparing means, our results showed a difference in proportions of low and high groups depending on sex, with augmented high P-gp activity group in men.

It has been previously demonstrated that P-gp expression has pronounced interindividual differences in various tissues such as small intestine and liver²².

Therefore, the role of ABCB1 genetic polymorphisms or haplotypes on P-gp expression and its relation with pharmacokinetics and toxicology are currently areas of intensive research²³.

A possible relationship between the P-gp polymorphisms and the P-gp activity variability can not be ruled out, though it has been reported that measurements of Rhodamine efflux using flow cytometry from peripheral

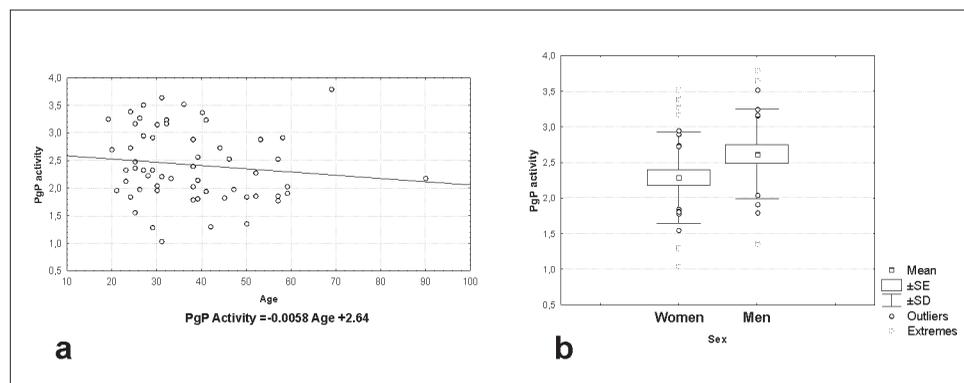


Fig. 4.– P-gp activity according to age and sex. P-gp activity is expressed as the ratio of mean fluorescence intensity for Rh123 as Rh123 + Verapamil/Rh123. (a) Relationship between volunteers' age and P-gp activity. Regression equation is shown, $r^2 = 0.015$. (b) Sex differences in mean P-gp activity. Mean and SD are shown $p > 0.05$ (Mann-Whitney U-test, two tailed).

blood cells allows assessment of genetically determined differences in P-gp function¹².

Many observations have been reported considering the impact of P-gp polymorphisms and haplotypes on therapeutics and susceptibility to several diseases, albeit, by the moment, a lot of discrepancies are found²³.

The regulation of lymphocytes uptake conducted by P-gp may be determinant in some therapies because appropriate intracellular concentrations are required for therapeutic agents as antiviral drugs²⁴.

Herein, results of our laboratory showed an inverse correlation between P-gp lymphocytic activity and the bioavailability of an oral Indinavir formulation²⁵. If this result is confirmed, an interesting parameter value can be obtained from blood, for a better therapeutic use of some drugs substrate of P-gp.

Between the great numbers of drug transporters, P-gp has been the best studied and the one that has more tools for its evaluation. Within many techniques, Rh123 efflux assay seems to be a reliable tool to measure P-gp functionality²⁶. Besides, a good correlation between Rh123 efflux, P-gp expression and multidrug resistance type 1 mRNA level has been previously observed, indication that this assay provides a surrogate marker for P-gp expression²⁷. Moreover, peripheral blood cells represent an easily accessible sample from which we can obtain the valuable information of individual phenotypic P-gp function.

As it has been already observed, metabolizing enzymes have different activities dividing the whole population into "good" and "bad" metabolizers²⁸. Considering the information reported in the literature and according to our results, it becomes possible to suggest that P-gp activity may also have two differentiated groups of high and low values within the population studied.

Characterization of P-glycoprotein functionality in the population represents a useful contribution to the begin-

ning of pharmacological treatments that consider its effect on pharmacokinetics and pharmacodynamics of individualized patients.

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Conflict of interests: There are no conflicts of interest.

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A un médico

*Yacen de un home en esta piedra dura
el cuerpo yermo y las cenizas frías:
Médico fue, cuchillo de natura,
Causa de todas las riquezas mías.*

*Y ahora cierro en honda sepultura
Los miembros que rigió por largos días;
Y aun con ser Muerte yo, no se la diera,
Si dél para matarle no aprendiera.*

Francisco de Quevedo y Villegas (1580-1645)

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