# Examination of human membrane proteins in diseases – application of molecular cell biology to study the SARS-CoV-2 spike protein

Ph.D. thesis

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Budapest, 2025

# 1. Introduction

Membrane proteins play essential roles in life, acting as mediators of communication, transport, and protection across membranes. These proteins are vital in maintaining cellular health and are implicated in various diseases, making them important therapeutic targets. Among the diverse types of membrane proteins, this dissertation focuses on three significant examples: the ABCG2 multidrug transporter, the PMCA4b calcium transporter, and the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein.

The ABCG2 transporter is critical in drug resistance and pharmacokinetics. As part of the ATP-binding cassette (ABC) transporter family, it helps export diverse substrates, including drugs and toxins, from cells. Genetic variants of ABCG2, such as the common Q141K variant, affect its expression and function, influencing drug efficacy and disease risk, and increases gout susceptibility.

PMCA4b, the plasma membrane calcium ATPase encoded by the *ATP2B4* gene, is integral to cellular calcium regulation. It maintains low calcium levels in red blood cells, and its reduced function is associated with resistance to severe malaria. This dissertation explores how SNPs in the malaria-associated haplotype regulate *ATP2B4* expression in an erythroid-specific way and result in the reduced PMCA4b protein expression.

Finally, the SARS-CoV-2 spike protein's RBD is crucial in COVID infection, as mutations in this domain contribute to immune escape, complicating vaccine efficacy and immunity assessment. This work examines antibody responses to various RBD variants, providing insights into immunity after vaccination or illness across emerging strains.

Through these proteins, this research highlights the impact of genetic variation on membrane protein function, with implications for personalized medicine, infectious disease management, and therapeutic development.

## 2. Objectives

My aims were to

- **Explore the consequences of the K360del (rs750972998) ABCG2 variant**: Investigate the impact of the variant on protein expression and function, and the role of the unstructured cytoplasmic loop affected by this variant.
- **Examine the effect of a haplotype in a regulatory region of the *ATP2B4* gene**: Study the effect of a haplotype in a supposed regulatory region of the *ATP2B4* gene encoding the PMCA4b calcium pump.
- **Generate a stable HEK293 suspension cell line**: Develop a stable HEK293 suspension cell line to produce the SARS-CoV-2 spike and spike RBD proteins secreted into the serum-free cell culture media.
- **Produce the RBD proteins of newly emerging SARS-CoV-2 variants:** Produce RBD proteins according to the altered sequence of newly emerging SARS-CoV-2 variants and use them in an ELISA to evaluate the anti-spike-RBD antibody levels of vaccinated individuals and COVID-19 patients.

The research process included the identification of the best molecular biological and cellular biological methods to examine the effects of clinically relevant variants. The production of the RBD protein was achieved using the same stable cell line generation method applied in the other experiments.

# 3. Methods

## 3.1. Vector constructs

The p10-[protein coding gene]-IRES2-GFP transposon vector for expressing untagged and tagged ABCG2 and spike proteins was used. Mutations were introduced via PCR and cassette replacement, and all vector sequences were validated by Sanger sequencing. Stable cell line generation utilized the Sleeping Beauty transposon-transposase system, ensuring reliable integration of the p10 vector with the SB100 transposase. For transient expression experiments, only the p10 vector was used. For dynamic trafficking, RUSH vectors with tagged ABCG2 variants were used, incorporating an ER hook as described in Bartos and Homolya (2021). Specific mutations were introduced by replacing the WT-ABCG2 coding sequence in these plasmids, verified by sequencing.

## 3.2. Cell culture and transfection

Multiple cell lines, including HEK293, HeLa, MDCKII, K562, and HEL, were cultured in appropriate media with 10% FBS and 1% Penicillin-Streptomycin. HEK293 and HeLa cells were transfected using Lipofectamine 2000, while K562 and HEL cells utilized Mirus TransIT. For serum-free culturing, HEK293 cells were adapted to FreeStyle293 medium.

Cycloheximide treatments in ABCG2 variant experiments used a final concentration of 20 μg/mL for 1-4 hours. Stable cell lines were generated by transposon-transposase-mediated integration, sorted for GFP fluorescence and overexpression of target proteins.

## 3.3. Stable cell line generation

Stable lines were established via the Sleeping Beauty transposon-transposase system, selecting cells with high GFP expression indicative of successful integration. Sorted MDCKII cells produced mixed cultures for polarized localization studies,

while HEK293 cells expressing spike variants underwent single-cell sorting.

### 3.4. Dual-luciferase assay

To assess promoter activity of the *ATP2B4* haplotype-affected regions, dual-luciferase assays were conducted on HEK293, HEL, and K562 cells. Results were normalized to control samples.

### 3.5. RNA isolation and cDNA PCR

RNA isolation from HEK293, HEL, and K562 cells was performed to study GATA-1 mRNA expression, using GATA-1-specific primers and Phusion High Fidelity polymerase.

### 3.6. Protein purification from culture media

HEK293 cell lines expressing spike-RBD protein were cultured in suspension and serum-free media. Protein was purified by nickel affinity chromatography and confirmed by SDS-PAGE and spectrophotometry.

### 3.7. Hoechst33342 uptake assay

ABCG2 function was assessed by measuring Hoechst33342 dye uptake in live cells overexpressing ABCG2 variants. The ABCG2 inhibitor Ko143 confirmed transporter activity. Uptake data were analyzed to calculate multidrug resistance activity factors (MAF).

### 3.8. ABCG2 surface expression

ABCG2 cell surface expression was measured using flow cytometry with the conformation-sensitive 5D3 antibody, following cell labeling with primary and secondary antibodies. Ko143 was used to enhance antibody recognition.

### 3.9. Western blotting

Protein samples from various experiments were analyzed by Western blot, detecting ABCG2, GATA1, and spike proteins using specific primary antibodies and HRP-conjugated secondary antibodies. Luminescence was detected with a

ChemiDoc imaging system, and ImageJ software was used for densitometry analysis.

### 3.10. Immunostaining and confocal imaging

MDCKII cells cultured on Transwell membranes were immunostained for confocal imaging, utilizing antibodies against ABCG2 and basolateral markers. Cells were visualized using a Zeiss confocal microscope, and images were processed with ZEN software.

### 3.11. Kinetic analysis of ABCG2 trafficking

The RUSH system facilitated analysis of ABCG2 trafficking dynamics in HeLa cells. Cells were labeled with biotin and specific antibodies, then observed over time via confocal microscopy. Localization coefficients were calculated and fitted to a sigmoidal function for kinetic analysis.

### 3.12. ELISA for antibody detection against the SARS-CoV-2 RBD

An indirect ELISA was optimized to detect SARS-CoV-2 RBD-specific antibodies in human sera. Serum dilutions were analyzed with coated RBD, anti-RBD antibodies, and TMB substrate for colorimetric detection. Absorbance results were detected by a plate reader and visualized using GraphPad software.

### 3.13. Statistical analysis

All data were analyzed in GraphPad Prism using ANOVA and Dunnett's test for comparisons to WT-ABCG2. ), Student's t-test was used when comparing dual-luciferase results. Significant differences were marked * ($p < 0.05$).

# 4. Results

## 4.1 Examination of variants in a cytoplasmic loop of the ABCG2 multidrug transporter

### *4.1.1. Structure and mutagenesis of the ABCG2 cytoplasmic loop*

This study focused on a cytoplasmic loop within the ABCG2 transporter, a region comprising amino acids 354-367. This loop includes four lysines (K357-K360) and a threonine (T362), which may be phosphorylated and serve a regulatory role. Variants examined included the deletion of K360 (K360del), individual and combined lysine-to-alanine substitutions, and replacements of threonine with alanine (T362A) and glutamic acid (T362E) to explore the functional effects of these amino acids.

### *4.1.2. Expression, membrane localization, and function of the examined ABCG2 variants*

ABCG2 variant expression and localization were tested in HeLa and HEK293 cells transfected with untagged ABCG2 constructs. A co-expressed GFP allowed for assessing expression efficiency, while an antibody targeting ABCG2's extracellular domain was used to evaluate cell surface localization. All ABCG2 variants, including lysine and threonine mutations, displayed surface expression levels similar to the wild-type (WT) protein.

Western blot analysis confirmed ABCG2's presence across variants, though some showed slight differences in overall cellular expression. Interestingly, the T362 to alanine variant did not show altered or decreased expression or function, in contrast to a previous result in the literature.

Functional assays measuring the transport of Hoechst33342 dye, a known ABCG2 substrate, demonstrated that all variants retained the protein's transport capability. Multidrug resistance

activity factors (MAF) for the variants were largely comparable to WT.

#### *4.1.3. Polarized localization in MDCKII cells*

The study further evaluated the impact of lysine modifications on ABCG2's polarized localization in MDCKII cells, a polarized cell model. ABCG2 variants were tested for apical membrane localization, typical of the WT protein in polarized cells. All lysine variants were successfully positioned at the apical membrane, suggesting that lysine modifications within this loop do not impact ABCG2's polarized distribution in this cellular model.

#### *4.1.4. Summary*

The results suggest that all examined ABCG2 variants reach the cell surface and maintain functional transporter activity. Even when replacing the lysine cluster with alanines, the transporter exhibited proper membrane expression, full activity, and apical membrane localization, indicating the resilience of this loop region. Interestingly, modifications at the T362 phosphorylation site (T362A or T362E) did not significantly alter expression or function.

### 4.2 Examination of SNPs in a malaria-associated haplotype in the *ATP2B4* gene, and their effect on promoter activity

#### *4.2.1. Promoter activity of the regions affected by the haplotype*

The malaria-associated haplotype in the *ATP2B4* gene affects a region up-and downstream the 2$^{nd}$ exon, which was divided into four sequences for detailed analysis. Each sequence (WT and haplotype with the SNPs) was cloned into a luciferase reporter plasmid to assess promoter activity. These luciferase constructs were then transfected into erythroid cell lines (K562 and HEL92) and a non-erythroid cell line (HEK293) to evaluate promoter activity in different cellular contexts.

The luciferase signal measured in erythroid cells indicated that the "H1st" region has higher promoter activity in these cells than in HEK293 cells, demonstrating an erythroid-specific enhancer effect. However, when SNPs were present within this region, a marked decrease in promoter activity was observed in the erythroid cell lines, though no such reduction occurred in HEK293 cells. These results suggest that the ATP2B4 haplotype SNPs specifically reduce the promoter activity of the H1st region in erythroid cells, likely influencing PMCA4b expression in an erythroid-specific manner.

#### **4.2.2.** *Role of the GATA-1 transcription factor*

GATA-1, a key erythroid-specific transcription factor, was predicted to bind within the H1st sequence. cDNA PCR and Western blot confirmed the presence of GATA-1 in erythroid cell lines, but not in non-erythroid HEK cells. Cell lines were transfected with the GATA-1 coding sequence which resulted in GATA-1 overexpression, and the luciferase coding sequences to study promoter activity in GATA-1 overexpressing cells. Erythroid cells overexpressing GATA-1 exhibited enhanced H1st promoter activity in luciferase assays, and this effect was more pronounced in cells containing the wild-type *ATP2B4* sequences compared to the minor haplotype, suggesting GATA-1's role in modulating the decreased red blood cell PMCA4b expression in the haplotype-carriers.

#### **4.2.3.** *SNP-specific mutation analysis*

The study further examined the specific haplotype SNPs that disrupt predicted GATA-1 binding sites in the *ATP2B4* gene. Targeted mutations in the "H1st" region SNPs reduced gene expression in luciferase assays, indicating that these sites are critical for GATA-1-dependent regulation of PMCA4b in erythroid cells.

#### **4.2.4.** *Summary*

This section highlights the regulatory role of specific SNPs in the malaria-associated haplotype of the *ATP2B4* gene in erythroid cells where GATA-1 appears central to regulate expression. The findings emphasize the importance of GATA-1 binding sites and provide insight into the molecular regulation of haplotype-associated factors that may influence red blood cell calcium levels through decreasing PMCA4b expression.

## 4.3 Production and analysis of the SARS-CoV-2 spike RBD protein in an ELISA assay

### **4.3.1.** *Stable production of the SARS-CoV-2 spike RBD protein (and its variants) in HEK293 cells*

HEK293 cell lines stably expressing the SARS-CoV-2 spike RBD protein of the Wuhan and Omicron BA.1 and BA.5 variants. Using the Sleeping Beauty transposon-transposase method. Single-cell cloning resulted in the selection of cell lines expressing the RBD protein in high amounts. Cells were adapted to serum-free growth in the FreeStyle Expression medium, RBD was secreted into the culture medium. RBD protein expression was confirmed via Western blot and ELISA.

### **4.3.2.** *ELISA assay to determine anti-RBD antibody levels in the sera of vaccinated and convalescent individuals*

ELISA assays were optimized to measure anti-RBD IgG antibody levels in vaccinated individuals and COVID-19 patients. Key findings of the study:

- **Vaccinated individuals:**

There was a huge individual variation in antibody levels, with a general decline in IgG antibodies approximately six months after the last vaccine dose or latest infection. This finding confirms the fading of antibody levels over time post-vaccination or infection.

- **COVID-19 patients**

Among COVID-19 patients, anti-RBD IgG levels showed cross-reactivity against Omicron variants, even tough at the time of sample collection, this variant was not recorded. No significant differences could be observed in antibody levels across the different COVID-19 severity groups, although this study involved a limited number of patients. Huge individual variations existed among individuals, that could not be explained by the examined factors. There were patients in all severity groups with extremely elevated antibody levels.

#### **4.3.3.** *Summary*

The ELISA assay to measure anti-RBD IgG levels in human sera underscores the robustness of cross-reactive antibodies to Wuhan and Omicron variants. Antibody titers generally decreased over time in vaccinated individuals, highlighting the benefit of booster vaccinations. Meanwhile, the extremely high antibody levels observed in hospitalized COVID-19 patients of variable disease severity provide insight into immune response dynamics following natural infection.

## 5. Conclusions

This research provides valuable insights into the functional dynamics of the ABCG2 transporter, the erythroid-specific regulatory effects of SNPs in the *ATP2B4* gene on PMCA4b expression, and the development of a robust SARS-CoV-2 RBD-based assay to assess immune responses. Each of these findings has distinct clinical and biological implications:

**1. Clinical and biological relevance of ABCG2 transporter variants**

The study of ABCG2, particularly the naturally occurring K360del variant, reveals important aspects of transporter function and trafficking. This disordered cytoplasmic loop variant demonstrated enhanced trafficking to the plasma

membrane, resulting in higher surface expression levels of ABCG2. Understanding these trafficking dynamics has clinical implications, particularly for cancers and other diseases where ABCG2-mediated drug resistance limits therapeutic efficacy and protein expression in different key barriers influences drug availability and distribution. Variants that alter ABCG2's trafficking or surface expression can influence drug efflux, potentially impacting drug sensitivity and treatment outcomes in patients.

Moreover, the T362A mutation, which showed normal function despite lacking the suggested regulatory phosphorylation site, raises questions about T362 phosphorylation's role in transporter regulation. Given ABCG2's relevance in drug distribution, this finding suggests that alternative regulatory mechanisms, potentially independent of phosphorylation, may exist. These results highlight the importance of trafficking, and how it can influence ABCG2 cell surface expression levels. As decreased ABCG2 expression levels that result in decreased ABCG2 function have been linked to susceptibility to gout, it is important to classify naturally occurring, missense mutations in ABCG2. The K360del variant should not be considered a decreased function variant.

## 2. Impact of *ATP2B4* gene SNPs on erythroid-specific PMCA4b expression and malaria resistance

The *ATP2B4* gene, encoding the PMCA4b calcium transporter, is shown here to be regulated in an erythroid-specific manner through a haplotype-affected promoter region. The SNPs within this region, associated with malaria resistance, demonstrated a marked reduction in promoter activity when present, in the H1st regulatory region in erythroid cells. PMCA4b red blood cell expression is decreased in the carriers of this minor haplotype. These results examined the molecular mechanisms behind the decrease in expression.

By identifying SNPs that reduce erythroid-specific promoter activity, this work may contribute to developing genetic screening tools in malaria-prone regions. Screening for these specific SNPs could allow early identification of individuals with altered erythrocyte calcium homeostasis, which may confer a degree of protection against malaria. Furthermore, understanding how PMCA4b expression modulates erythrocyte susceptibility to malaria opens doors to therapeutic interventions aimed at manipulating PMCA4b levels as a protective mechanism in endemic areas, potentially providing a novel angle in malaria prevention strategies.

**3. SARS-CoV-2 RBD production for immune response monitoring**

The successful production of the SARS-CoV-2 spike RBD protein, including Omicron and Wuhan variants, in suspension HEK293 cells enables scalable testing of antibody responses in vaccinated individuals and COVID-19 patients. The ELISA developed in this work provides a simple yet effective method to monitor IgG antibody levels against different SARS-CoV-2 variants.

From a clinical perspective, the ability to measure variant-specific antibody responses is crucial as the virus continues to evolve. With emerging variants often displaying mutations in the RBD, the assay developed here provides a way to evaluate immune protection at an individual level, informing decisions on the need for booster doses or updated vaccines. This approach could be particularly beneficial in tracking immunity in vulnerable populations and evaluating the efficacy of new vaccine formulations as they become available.

Biologically, these findings underscore the importance of immune monitoring for pandemic management. The assay can be adapted for future variants, positioning it as a versatile tool for real-time epidemiological studies and guiding public health

responses. Additionally, the scalability of the HEK293 cell system for RBD production enhances the potential for widespread application in clinical and research settings, supporting efforts to manage COVID-19 through tailored immunization strategies.

**Potential impact and future directions**

The outcomes of this research contribute to potential applications in disease management and therapeutic development:

- **pharmacokinetics :** The findings on ABCG2 trafficking and T362 phosphorylation-independent regulation may influence future drug design. In predicting drug-drug interactions, carriers of the K360del variant should be considered as carriers of a normally functioning ABCG2.
- **in malaria:** By identifying specific SNPs that impact *ATP2B4* expression in red blood cells, and the potential transcription factor responsible for the altered expression, we are getting closer to understanding the molecular-level impact of protection against malaria. This should help in the design of novel drug candidates to treat or even prevent the disease.
- **in COVID:** The SARS-CoV-2 RBD ELISA assay exemplifies how rapid production and application of viral antigens can support real-time monitoring of immune protection across variants. As SARS-CoV-2 continues to mutate, such diagnostic tools will be essential for tracking immunity and guiding public health decisions on vaccination and booster strategies.

Together, these findings should contribute to the understanding and managing membrane protein variants in clinical contexts, from improving personalized drug treatments to enhancing malaria treatment and responding to viral outbreaks. This research highlights the importance of variant-specific studies in

advancing personalized and population-level, up-to-date health interventions.

## 6. Bibliography of the candidate's publications

**Publications related to the thesis:**

**Mózner, Orsolya** ; Zámbó, Boglárka ; Bartos, Zsuzsa ; Gergely, Anna ; Szabó, Kata Sára ; Jezsó, Bálint ; Telbisz, Ágnes ; Várady, György ; Homolya, László ; Hegedűs, Tamás ; Balázs Sarkadi
Expression, Function and Trafficking of the Human ABCG2 Multidrug Transporter Containing Mutations in an Unstructured Cytoplasmic Loop
**MEMBRANES (BASEL)** 13 : 10 Paper: 822 , 14 p. (2023)
**IF: 3.3**
DOI: 10.3390/membranes13100822

**Mózner, Orsolya*** ; Zámbó, Boglárka*; Sarkadi, Balázs
Modulation of the Human Erythroid Plasma Membrane Calcium Pump (PMCA4b) Expression by Polymorphic Genetic Variants
**MEMBRANES (BASEL)** 11 : 8 Paper: 586 , 10 p. (2021)
**IF: 4.562**
DOI: 10.3390/membranes11080586

**Mózner, Orsolya** ; Moldvay, Judit ; Szabó, Kata Sára ; Vaskó, Dorottya ; Domján, Júlia ; Ács, Dorottya ; Ligeti, Zoltán ; Fehér, Csaba ; Hirsch, Edit ; Puskás, László ; Stahl, Cordula ; Frey, Manfred ; Sarkadi, Balázs
Application of a Receptor-Binding-Domain-Based Simple Immunoassay for Assessing Humoral Immunity against Emerging SARS-CoV-2 Virus Variants
**BIOMEDICINES** 11 : 12 Paper: 3193 , 10 p. (2023)
**IF: 3.9**
DOI: 10.3390/biomedicines11123193

**Publications not related to the thesis:**

Papp H, Tóth E, Bóvári-Biri J, Bánfai K, Juhász P, Mahdi M, Russo LC, Bajusz D, Sipos A, Petri L, Szalai TV, Kemény Á, Madai M, Kuczmog A, Batta Gy, **Mózner O**, Vaskó D, Hirsch E, Bohus P, Méhes G, Tőzsér J, Curtin NJ, Helyes Zs, Tóth A, Hoch NC, Jakab F, Keserű GYM, Pongrácz JE, Bai P
The PARP inhibitor rucaparib blocks SARS-CoV-2 virus binding to cells and the immune reaction in models of COVID-19
**BRITISH JOURNAL OF PHARMACOLOGY** DOI: 10.1111/bph.17305, 22 p. (2024)
**IF: 6,8#**

Vaskó Dorottya, Pantea Eszter, Domján Júlia, Fehér Csaba, **Mózner Orsolya**, Sarkadi Balázs, Nagy Zsombor, Marosi György J, Hirsch Edit
Raman and NIR spectroscopy-based real-time monitoring of the membrane filtration process of a recombinant protein for the diagnosis of SARS-CoV-2
**INTERNATIONAL JOURNAL OF PHARMACEUTICS** 660 Paper: 124251, 10 p. (2024)
**IF: 5,3#**

Broca-Brisson Léa, Harati Rania, Disdier Clémence, **Mózner Orsolya,** Gaston-Breton Romane, Maïza Auriane, Costa Narciso, Guyot Anne-Cécile, Sarkadi Balázs, Apáti Ágota, Skelton Matthew R, Madrange Lucie, Yates Frank, Armengaud Jean, Hamoudi Rifat, Mabondzo Aloise
Deciphering neuronal deficit and protein profile changes in human brain organoids from patients with creatine transporter deficiency
**ELIFE** 12 Paper: RP88459, 22 p. (2023)

**IF: 6,4**

Pálinkás M, Szabó E, Kulin A, **Mózner O**, Rásonyi R, Juhász P, Nagy K, Várady G, Vörös D, Zámbó B, Sarkadi B, Poór Gy
Genetic polymorphisms and decreased protein expression of ABCG2 urate transporters are associated with susceptibility to gout, disease severity and renal-overload hyperuricemia
**CLINICAL AND EXPERIMENTAL MEDICINE** 23: 4 pp. 1277-1284. (2023)
**IF: 3,2**

Szabó Edit, Kulin Anna, **Mózner Orsolya**, Korányi László, Literáti-Nagy Botond, Vitai Márta, Cserepes Judit, Sarkadi Balázs, Várady György
Potential role of the ABCG2-Q141K polymorphism in type 2 diabetes
**PLOS ONE** 16: 12 Paper: e0260957, 9 p. (2021) Q1
**IF: 3,752**

Telbisz Ágnes, Ambrus Csilla, **Mózner Orsolya**, Szabó Edit, Várady György, Bakos Éva, Sarkadi Balázs, Özvegy-Laczka Csilla
Interactions of Potential Anti-COVID-19 Compounds with Multispecific ABC and OATP
Drug Transporters
**PHARMACEUTICS** 13: 1 Paper: 81, 18 p. (2021)
**IF: 6,525**

Zámbó B*, **Mózner O***, Bartos Z, Török G, Várady G, Telbisz Á, Homolya L, Orbán TI, Sarkadi B
Cellular expression and function of naturally occurring variants of the human ABCG2 multidrug transporter
**CELLULAR AND MOLECULAR LIFE SCIENCES** 77: 2 pp. 365-378. (2020)

**IF: 9,261**

Zámbó B, Bartos Z, **Mózner O**, Szabó E, Várady G, Poór G, Pálinkás M, Andrikovics H, Hegedűs T, Homolya L, Sarkadi B
Clinically relevant mutations in the ABCG2 transporter uncovered by genetic analysis linked
to erythrocyte membrane protein Expression
**SCIENTIFIC REPORTS** 8: 1 Paper: 7487, 13 p. (2018)
**IF: 4,011**

*: shared first authors
#: expected impact factor

**∑IF: 57,011**