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Corresponding Author: Young Yil Bahk

Authors: Young Yil Bahk[1,*], Sang Bong Lee[2], Sang Bong[1], Jong Bo Kim[3], Jong Bo[1], Tong Soo Kim[4], Tong Soo[3], Sung Jong Hong[5], Sung Jong[3], Sung Kyu Ahn[6], Dong Min[4], Dong Min Kim[7], Sungkeun [5], Sungkeun Lee[8]

Institution: [1]Dept. of Biotechnology, Konkuk University Glocal Campus, [2]Research Institute for Biomedical & Health Science, Konkuk University,

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Elevated Plasma α1-Antichymotrypsin is a Biomarker Candidate for Malaria Patients.

(Running title: A biomarker candidate for malaria patient)

Young Yil BAHK[1,2*], Sang Bong LEE[1], Jong Bo KIM[1], Tong-Soo KIM[3] , Sung-Jong HONG[3], Dong Min KIM[4], Sungkeun LEE[5]

[1]Department of Biotechnology, College of Biomedical and Health Science and [2]Research Institute for Biomedical & Health Science, Konkuk University, Chungju 27478, Korea; [3]Convergence Research Center for Insect Vectors, Incheon National University, Incheon 22012, Korea; [4]Department of Internal Medicine, College of Medicine, Chosun University, Kwangju 61452, Korea; [5]Department of Pharmacology, Inha University School of Medicine, Incheon 22212, Korea.

*** Corresponding author:**

Young Yil BAHK, PhD

Department of Biotechnology,

College of Biomedical and Health Science

Konkuk University, Chungju 27478, Korea

E-mail: bahk12@empal.com

Tel: +82-43-840-3903

Abstract

Advancements in the field of proteomics have provided opportunities to develop diagnostic and therapeutic strategies against various diseases. About half of the world's population remains at risk of malaria. Caused by protozoan parasites of the genus *Plasmodium*, malaria is one of the oldest and largest risk factors responsible for the global burden of infectious diseases with an estimated 3.2 billion persons at risk of infection. For epidemiological surveillance and appropriate treatment of individuals infected with *Plasmodium* spp., timely detection is critical. In this study, we used combinations of depletion of abundant plasma proteins, 2-dimensional gel electrophoresis (2-DE), image analysis, LC-MS/MS and western blot analysis on the plasma of healthy donors (100 individuals) and vivax and falciparum malaria patients (100 vivax malaria patients and 8 falciparum malaria patients). These analyses revealed that α1-antichymotrypsin (AACT) protein levels were elevated in vivax malaria patient plasma samples (mean fold-change ± standard error: 2.83±0.11, based on band intensities), but not in plasma from patients with other mosquito-borne infectious diseases. The results of AACT immunoblot analyses showed that AACT protein was significantly elevated in vivax and falciparum malaria patient plasma samples (≥ 2-fold) compared to healthy control donor plasma samples, which has not been previously reported.

INTRODUCTION

Malaria is a significant public health burden with more than 3.2 billion people being at risk of infection in tropical and subtropical regions, especially in the resource-poor settings [**1**]. In 2020, 241 million malaria cases and 627,000 deaths were reported globally. In humans, malaria is exclusively caused by infection with a *Plasmodium* species (*Plasmodium falciparum, P. vivax, P. malaria, P. ovale*, or *P. kwowlesi*) that is transmitted to humans through the bite of infected female *Anopheles* mosquitoes [**1**-**3**]. Since the re-emergence of infectious vivax malaria in 1993, Korea has made significant progress in eliminating vivax malaria and has been consequently categorized as one of 32 malaria-eliminating countries [**4**]. Despite enormous control efforts, morbidity and mortality caused by malaria remain high in many developing countries, especially in areas characterized by tropical and subtropical ecosystems [**5**-**8**]. Malaria is a preventable and treatable condition [**9**].

Prompt and accurate diagnosis of malaria alongside reliable identification of *Plasmodium* species is pivotal for rapid and effective disease management, as a late diagnosis can result in significant morbidity and mortality. To eliminate malaria, more sensitive diagnostic tools should be provided to detect asymptomatic and sub-microscopic infections that contribute to transmission [**10**]. Accurate diagnosis and appropriate treatment of malaria are the keys to global malaria elimination, but the lack of accurate diagnostic tools has led to poor prognosis and delayed treatment [**11**]. Recently, omics-driven technologies have represented as an advancement in diagnostics and cell-based diagnosis, immune-chromatographic tests for parasitic proteins including *p*HRPII, *p*LDH, and aldolase, and mass-spectrometry based proteomics for host and parasite proteins [**12**, **13**]. Omics encompasses robust technique for approaching a biological problem from varying points of reference [**14**]. However, multi-omics or integrated omics remains a challenge in malaria detection, therefore optical microscopy still remains the gold standard technology for malaria diagnosis in most parts of the malaria-endemic world [**14**, **15**].

Traditional proteomic accesses can elucidate protein expression profiles, which may have useful

applications to clinical events, therapeutic responses, or investigation of the underlying mechanisms of diseases such as autoimmune disorders, cardiovascular diseases, and cancers [**16**, **17**]. Biomedical proteome research aimed at biomarker discovery is mainly based on expression proteomics, which analyzes the quantity of certain proteins in different conditions. Thus, proteomic studies are likely to be key factors in propelling the discovery of novel biomarkers.

With this goal, in this study, we initially analyzed plasma proteins of patients with vivax malaria alongside those of healthy specimen to discover biomarkers for the discrimination between long-term and short-term latent malaria. We also sought to acquire an in-depth understanding of the pathophysiological mechanism of the disease by using combinations of depletion of abundant proteins, 2-dimensional gel electrophoresis (2-DE), image analysis, and mass spectrometry. Though it failed to discriminate between long-term and short-term latent malaria, a plasma glycoprotein, α1-antichymotrypsin (AACT) could be developed as a possible biomarker for *Plasmodium* malaria, but not for other mosquito-borne infectious diseases.

RESULTS

To identify novel serologic biomarker candidates for malaria, we used plasma samples obtained from healthy donors and patients confirmed to be infected with vivax malaria. A typical 2-DE pattern of plasma from which 14 highly abundant proteins were removed as described previously is shown in **Supplementary Fig. 1A**. Although the primary goal of this study was to discover biomarkers to discriminate between long-term (L group) and short-term (S group) latent malaria, no differentially expressed proteins could be found in this study that would distinguish these two latent types of malaria. We excluded those proteins that are already known or involved in other diseases as well as those for which commercial antibodies are not available. Thus, AACT which was overexpressed in malaria plasma samples (2.5-fold in the L group compared with the control group, 2.6-fold in the S group compared with the control) was chosen as the sole candidate protein (**Supplementary Fig. 1B**). To verify AACT as a vivax malaria biomarker candidate and to assess its efficiency and specificity in diagnosing vivax malaria, we used 200 individual plasma samples from healthy donors (n=100) and vivax malaria patients (n=100); specifically, we evaluated differential AACT expression Western blot analysis, using a specific monoclonal antibody against AACT. As shown in **Fig. 1**, and **Supplementary Fig. 2**, the protein expression level of AACT in vivax malaria-patient samples was more than 2-fold higher than that in the control (means±standard errors, Control: 1±0.03 and vivax malaria patient: 2.83 ±0.11, based on band intensities, **Fig. 1D**). These results indicates that AACT is highly expressed in the plasma of vivax malaria patients. Equal loading (30 μg) of each sample on the gel was confirmed by staining with homemade Coomassie brilliant blue G250 dye. Malaria, which is transmitted by female *Anopheles* mosquitoes, results in peripheral blood parasitaemia which may manifest asymptomatically in adults living in malaria endemic areas [**18**]. The patients whose plasma was used for Western blot analysis had parasitaemia with between 20 and 28,381 parasites/μl (means±standard deviations, 3,504.9±6,159.1 parasites/μl). It is well-established that optical microscopy is able to identify parasite

103 species and determine parasite densities. However, the limit of detection by thick film microscopy is in
104 the range of 5 to 100 parasites/ml of blood [**19**, **20**]. It also requires experienced technicians and a
105 microscope, which are not always available in remote areas. Despite differences in their parasitaemia
106 of vivax patients, the AACT band intensities produced from Western blot analysis did not differ
107 according to the severity of parasitaemia (**Fig. 2**). According to the Korea Disease Control and
108 Prevention Agency, *P. falciparum* infections occurring in Korea were imported rather than domestic
109 cases [**21**, **22**]. In this study, eight imported falciparum malaria-patient plasma samples were also
110 subjected to Western blot analysis with the anti-AACT antibody. The protein expression level of AACT
111 in falciparum malaria samples was more than 2-fold higher than that in control samples, indicating that
112 AACT is also highly expressed in the plasma of falciparum malaria patients (**Fig. 3**). In practice, there
113 is no evidence-based information to guide clinicians in giving presumptive treatment when malaria is
114 strongly suspected, and laboratory confirmation is not immediately available [**23**].

115 Next, to investigate the relative expression level of AACT in other mosquito-borne infectious
116 diseases, we evaluated the comparative diagnostic specificity of AACT for malaria and other mosquito-
117 borne infectious diseases including Tsutsugamushi disease and Dengue fever. We performed Western
118 blot analyses using16 Dengue fever patient plasma samples (**Fig. 4A**) and 16 Tsutsugamushi disease
119 patient plasma samples (**Fig. 4B**). As shown in **Fig. 4**, the expression pattern and level of AACT in
120 Tsutsugamushi disease and Dengue fever samples differed from that of vivax and falciparum malaria
121 plasma and was similar to that of healthy plasma.

DISCUSSION

Glycoprotein AACT is a protein ascribed to the serine proteinase inhibitors superfamily, also known as serpins [**24**]. AACT is involved in the acute phase response, inflammation, and proteolysis. Although AACT is mainly synthesized in the liver, after which it is secreted into the blood, it is also synthesized in the brain, mainly by astrocytes [**25**, **26**]. In the brains of patients with Alzheimer's disease, AACT has been reported to bind amyloid-β peptides found in plaque cores and blood vessels and the protein is overexpressed in the brain of Alzheimer's disease patients [**27**, **28**]. Its expression is regulated by proinflammatory cytokines including interleukin (IL)-1, oncostatin M, and complexes of IL-6, soluble IL-6 receptors, and transcriptional regulators such as nuclear factor 1-X and activator protein 1 [**29**]. However, AACT's potential as a biomarker for Alzheimer's disease is controversial. These proteins inactivate proteinases with a serine residue in their active site [**30**]. AACT is a major acute-phase reactant, and its concentration in plasma increases in response to trauma, surgery, and infection and its elevated level is widespread, but not universal [**31**, **32**]. The dysregulation of AACT and its glycosylation levels are associated with tumor progression and recurrence, and could be used as a biomarker for monitoring tumors, including in liver cancer, pancreatic cancer, lung cancer, ovarian cancer, and diffuse large B-cell lymphoma [**33**]. However, the expression changes, glycan modification, and biological functions of AACT remain elusive.

Our results might confirm the malaria-specific up-expression of AACT in vivax and falciparum malaria. Malaria is suspected clinically primarily on the basis of fever or a history of fever. There is no combination of signs or symptoms that reliably distinguishes malaria from other causes of fever, and diagnosis based only on clinical features has very low specificity and results in overtreatment. To guide rational use of antimalaria medicines, the focus of malaria diagnoses should be to identify patients who truly have malaria,. The two methods used routinely for parasitological diagnosis of malaria are optical microscopy and immunochromatographic rapid diagnostic tests (RDTs). Optical microscopy of stained blood smears has important advantages including low direct cost, high sensitivity, differentiation of

147 *Plasmodium* species, and determination of parasite densities. However, the accuracy of microscopy
148 examination can be awkward to maintain because of the need to train and supervise of laboratory staff.
149 On the other hand, using a finger-prick blood sample, RDTs detect parasite-specific antigens based on
150 the detection of HRP2, pan-specific or species-specific *Plasmodium* LDH or pan-specific aldolase [**2**].
151 Although these tests have advantages, including rapid provision, fewer requirements for training
152 personnel, and reinforcement of patient confidence, they also have many potential disadvantages; these
153 disadvantages include the panel detection score of at least 75% at 200 parasites/μl, the false positive
154 rate, the existence of lot-to-lot variation, the frequencies of HRP2 deletions, and the poor sensitivity for
155 detecting *P. malariae* and *P. ovale*. In this study, the expression level of AACR in either vivax malaria
156 or falciparum malaria-patient plasma was upregulated. This analysis involved 100 vivax malaria blood
157 samples and 8 falciparum malaria blood samples. Microscopy examination and RDTs lack sensitivity
158 for low-level parasitaemia. The lower limit of detection (LOD) for microscopy is between 50 and 500
159 parasites/μl depending on the microscopist's expertise [**34**, **35**] and 100 parasites/μl for *Pf*HRP2 based
160 *P. falciparum* RDTs [**36**]. Recently, a multiplex qPCR analysis was developed that has high sensitivity
161 and high negative predictive value even in the LOD range of 0.05 parasites/μl including all *Plasmodium*
162 species was developed [**37**]. However, this assay does not resemble a field deployable rapid test due to
163 its expense and the requirement of advanced laboratory infrastructure. Here, using proteomics-based
164 technology, we identified a novel glycoprotein biomarker candidate even in samples reflecting low-
165 level parasitaemia (20 parasites/μl in blood) at the expression level of 2.83±0.11 fold, based on Western
166 blot analysis band intensities. The test for AACT was not responsive to other mosquito-borne parasitic
167 infectious diseases including Tsutsugamushi disease and Dengue fever.

168 Although this study demonstrated that AACT could serve as a novel biomarker for malaria caused
169 by *P. vivax* and *P. falciparum*, there are some limitations. First, this study is a retrospectively designed
170 study showing the potential usefulness of preoperative plasma AACT to predict prognosis by using
171 small and heterogenous samples. To acquire more statistical power, a large sample size including other

types of malaria such as, *P. falciparum* as well as different life cycle stages (ring stage, trophozoite, schizont, and gametocyte) is necessary in future work. In addition, to qualify AACT as a novel biomarker for malaria, further studies of AACT's applications, such as its usefulness in detecting recurrence and monitoring treatment are required. In addition, because malaria can be transmitted by blood transmission between humans and is undoubtedly responsible for the majority of the world's transfusion transmitted diseases [**38**]. For these reasons, and because the availability of molecular diagnostic methods is still limited, AACT might be used as a novel biomarker candidate to obtain information about low parasitaemia malaria and to diagnose *P. vivax* and *P. falciparum* infections.

In conclusion, we mapped differently expressed proteins in the plasma of vivax malaria patients using proteomic techniques, which allowed us to identify changes in AACT levels. Although the changes in AACT are likely not specific to vivax malaria patients, our study suggested the methodological advances for a proteomic approach to examine plasma proteins in malaria patients. Further investigation into the biochemical and cellular mechanism of AACT in malaria infection is warranted. These findings have clinical implications with respect to the elucidation of *Plasmodium* spp. infection and improved diagnosis of malaria. These promising results suggest the potential of utilizing AACT as a biomarker to detect *Plasmodium* spp. including *P. vivax* and *P. falciparum* in blood samples.

MATERIALS and METHODS

Ethics statement and sample collection

This study was performed under the regulation of the Institutional Review Board Committee of Konkuk University (No. **7001355-202007-BR-386**). This research adhered to the tenets of the Declaration of Helsinki. The malaria patients' and healthy donors' plasma was obtained from the Global Resource Bank of Parasitic Protozoa Pathogens in Incheon National University. The vivax malaria patients' plasma was obtained from 100 Korean patients and the falciparum malaria patients' plasmas were from 8 Korean patients confirmed in the Inha University Hospital and Inha University Department of Tropical Medicine. The *Plasmodium*-negative blood samples were obtained from 100 healthy people from three administrative districts in Korea (Cheolwon, Hampyeong, and Bosung; randomized and blindly collected). Plasma from patients with Tsutsugamushi disease and Dengue fever confirmed in Chosun University and Inha University was also collected from the Global Resource Bank of Parasitic Protozoa Pathogens.

Sample preparation for proteomic analysis and protein identification

Originally, we categorized the plasma samples into three groups: i) healthy donor (C group), ii) patients for whom the outbreak time coincides with the mosquito activity period (S group), and iii) patients for whom the outbreak time does not coincide with the mosquito activity period (L group). To remove 14 highly abundant proteins (albumin, immunoglobulin G (IgG), IgM, IgA, α1-antitrypsin, transferrin, haptoglobin, α2-macroglobulin, fibrinogen, complement C3, α1-acid glycoprotein (orosomucoid), HDL (apolipoproteins A-I and A-II), and LDL (mainly apolipoprotein B)) from human plasma, the multiple affinity removal column system based on avian antibody-antigen interactions (Seppro® IgY14, Millipore Sigma, St. Louis, MO, USA) was routinely used according to the manufacturer's recommended protocols. To search for a novel serologic indicator candidate for malaria, we carried out an integrated proteomic analysis using pooled plasma from healthy donors (C group)

and vivax malaria patient groups (S and L groups) [**39**, **40**]. All procedures for the proteomic analysis, including 2-DE, image analysis, trypsin digestion, protein identification by LC-MS/MS, and data searches for protein identification were performed by Yonsei Proteome Research Center (Seoul, Korea) as previously described [**39**, **40**].

Validation of the target proteins

Validation of some differentially expressed protein candidates was performed by Western blot analysis with the commercially available specific antibody. Total protein concentrations of plasma samples were estimated using a bicinchoninic acid-based protein assay system (Pierce, Rockford, IL, USA). Immunoreactive proteins on the membrane were detected using ECL Plus Western blotting detection reagents (GeneCure, Norcross, GA, USA). To evaluate band intensities, bands on the X-ray-films were imaged and analyzed using the ChemiDoc™ XRS + System equipped with Image Lab Software™ (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were expressed as means±standard errors and analyzed by a Student's *t*-test. Statistical significance was accepted at $P < 0.05$. IBM SPSS Statistics ver. 27 (IBM, Somers, NY, USA) was used for all of the statistical analyses.

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CONFLICT OF INTEREST

The listed authors declare that they have no conflicts of interest regarding the publication of this article.

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FIGURE LEGENDS

Fig. 1. (A). AACT in the plasma of healthy donors and vivax malaria patients detected by chemiluminescence (Blot #1): H numbered lanes are healthy group (1-5) and P numbered lanes (1-5) are patients. (B). Expression level of AACT in specimens was measured by comparing the band intensities of malaria-patient samples with those of healthy donors. Data in Ave. lanes are presented as means±standard errors of five independent lanes. AACT expression differed significantly between plasma from healthy donors (#H1-#H5) and plasma from donors with vivax malaria (#P1-#P5; $P<0.01$). (C) Expression level of AACT in specimens was measured by comparing the band intensities of each patient sample with that of the healthy donors (Blot #1~#20). (D). $P<0.01$, compared with healthy donor (#H1-#H100) versus vivax malaria patients (#P1-#P100).

Fig. 2. Western blot analysis and SDS-PAGE of the plasma samples from individuals with parasitaemia and individuals infected with *P. falciparum*. Equal amount of whole plasma from patients with different severities of parasitaemia was subjected to Western blot analysis and SDS-PAGE.

Fig. 3. Western blot analysis and SDS-PAGE of the whole plasma from falciparum malaria patients. (A). Samples from three vivax malaria patient (*Pv*1-*Pv*3) and three healthy donor (H1-H3) were used for positive and negative controls. Blot #1 represents 4 falciparum malaria patients (*Pf*1-*Pf*4) and blot #2 represents *Pf*5-*Pf*8. (B). Expression level of AACT in specimens was measured by comparing the band intensities of each falciparum malaria patient sample with those of healthy donors as negative control and those of vivax malaria patients as positive control: blot #1 represents *Pf*1-*Pf*4 and blot #2 represents *Pf*5-*Pf*8.

Fig. 4. Western blot analysis and SDS-PAGE of whole plasma from other mosquito-borne disease with healthy donor (H1) and vivax malaria patient (*Pv*1) samples. (A). Western blot analysis and SDS-PAGE of Dengue fever patients (D1-D16). (B). Western blot analysis and SDS-PAGE of

355 Tsutsugamushi disease patients (T1-T16).

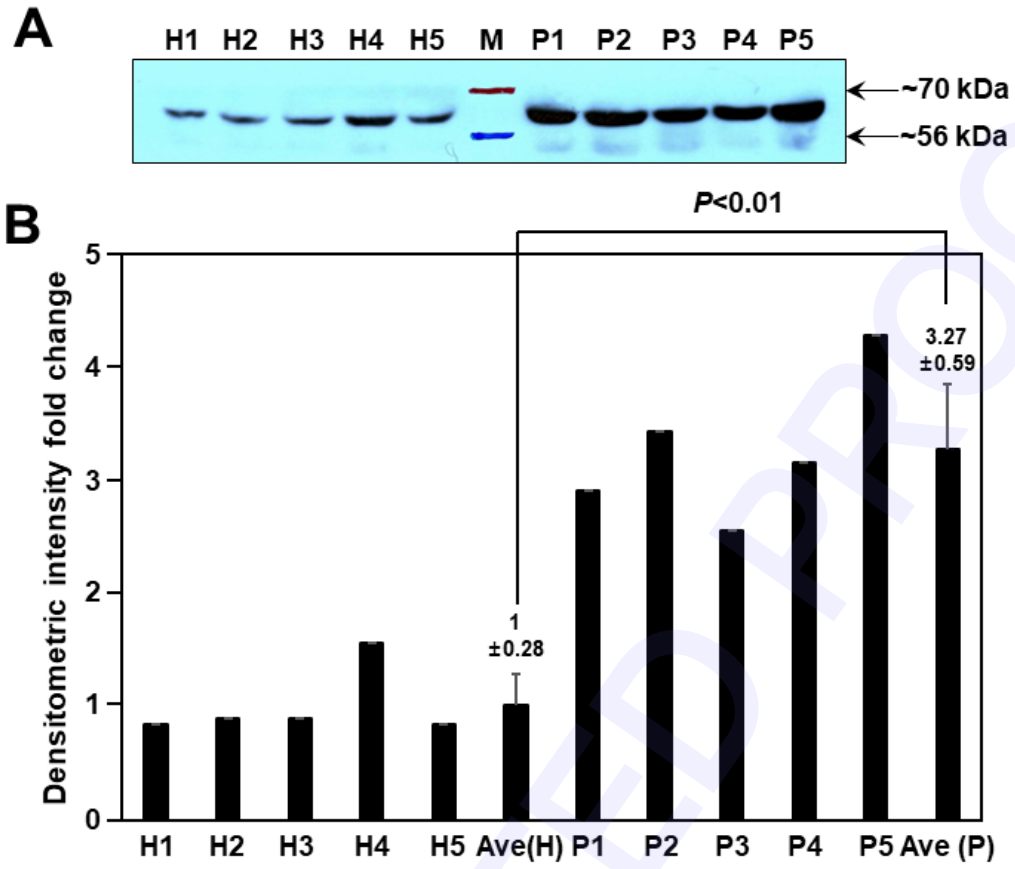


Fig. 1, Bahk et al.

Fig. 1. Fig 1A & B

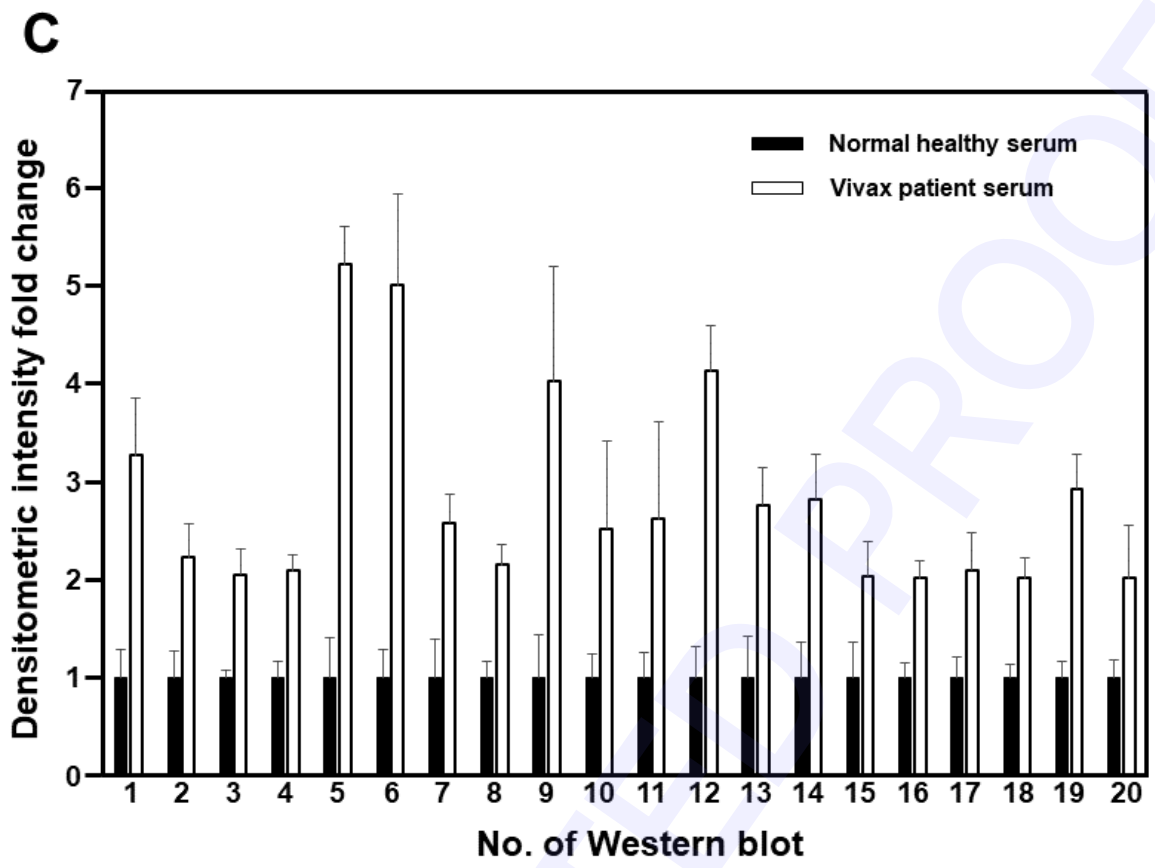


Fig. 1, Bahk et al.

Fig. 2. Fig. 1C

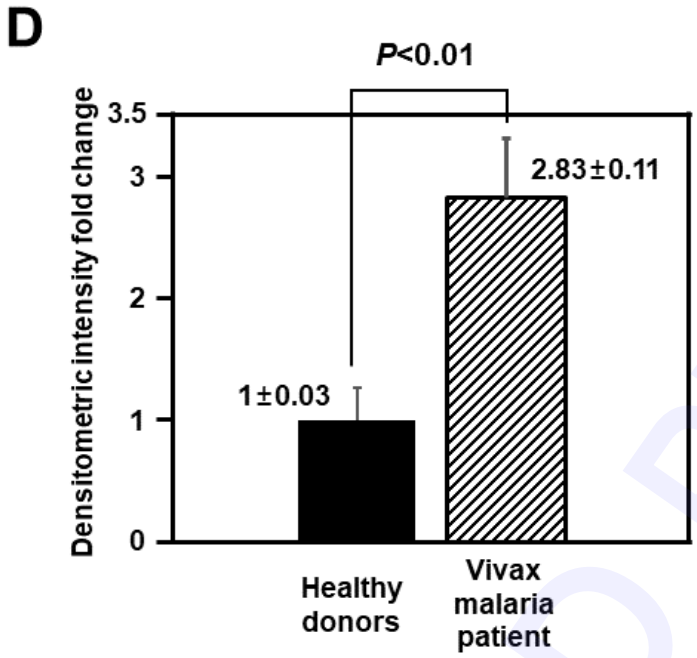


Fig. 1, Bahk et al.

Fig. 3. Fig. 1D

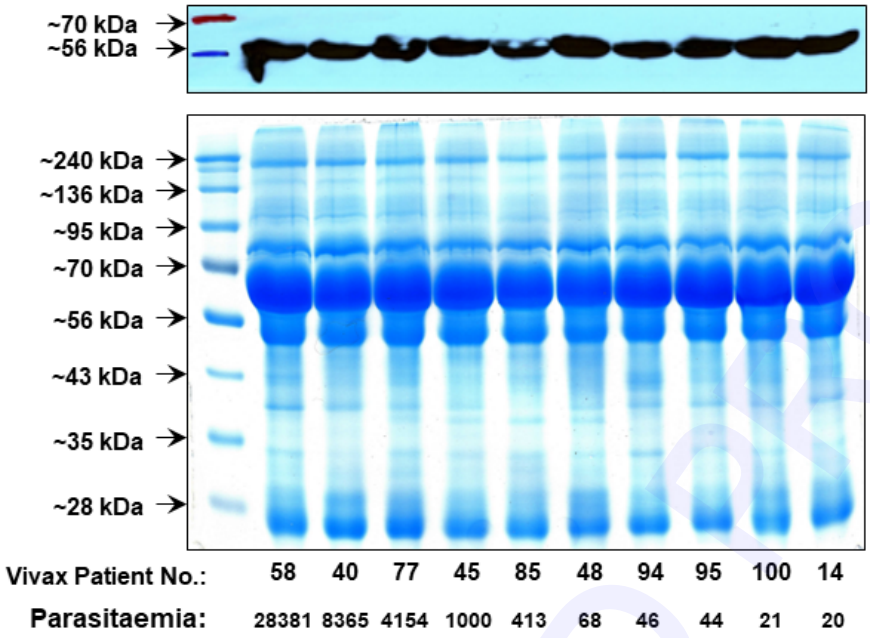


Fig. 2, Bahk et al.

Fig. 4. Fig. 2

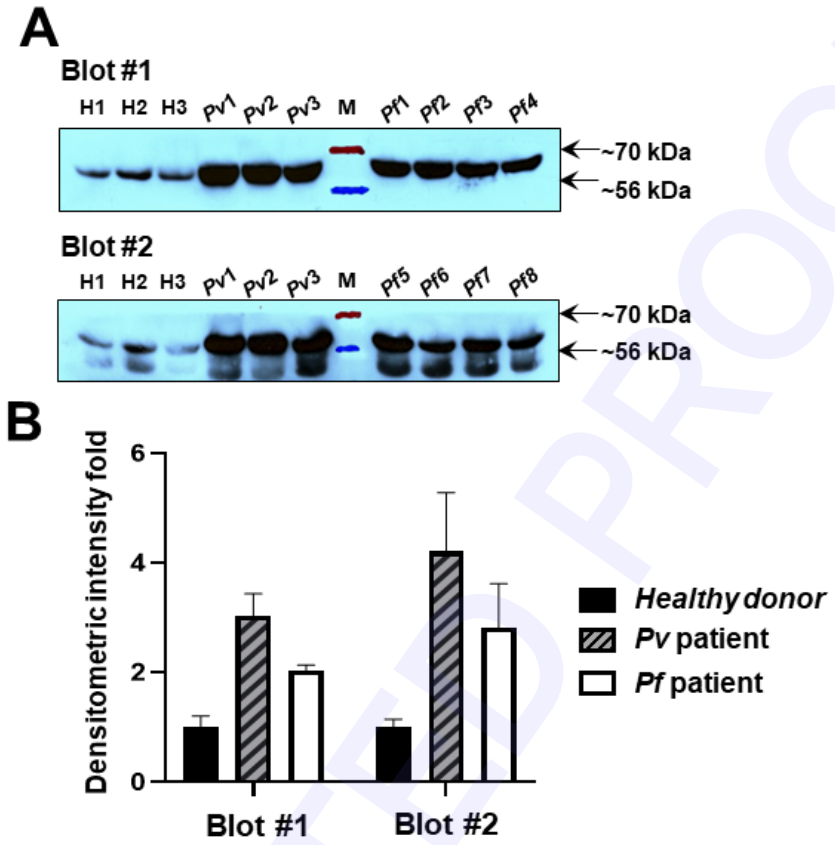


Fig. 3, Bahk et al.

Fig. 5. Fig. 3 A & B

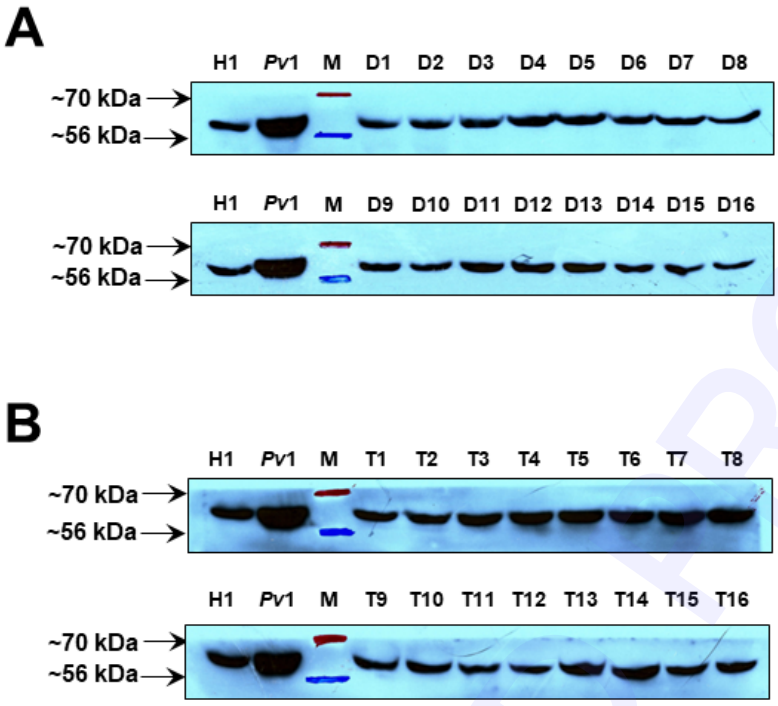
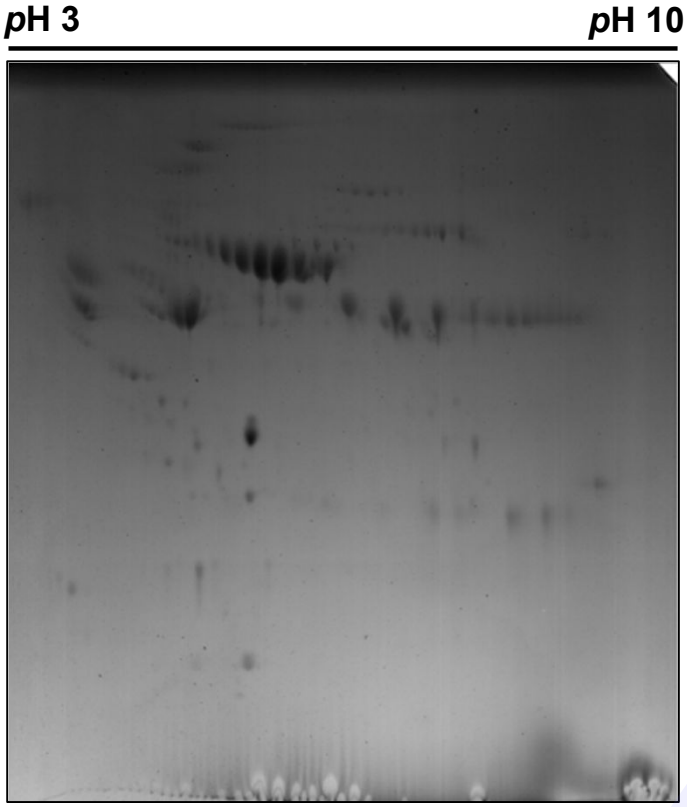


Fig. 4, Bahk et al.

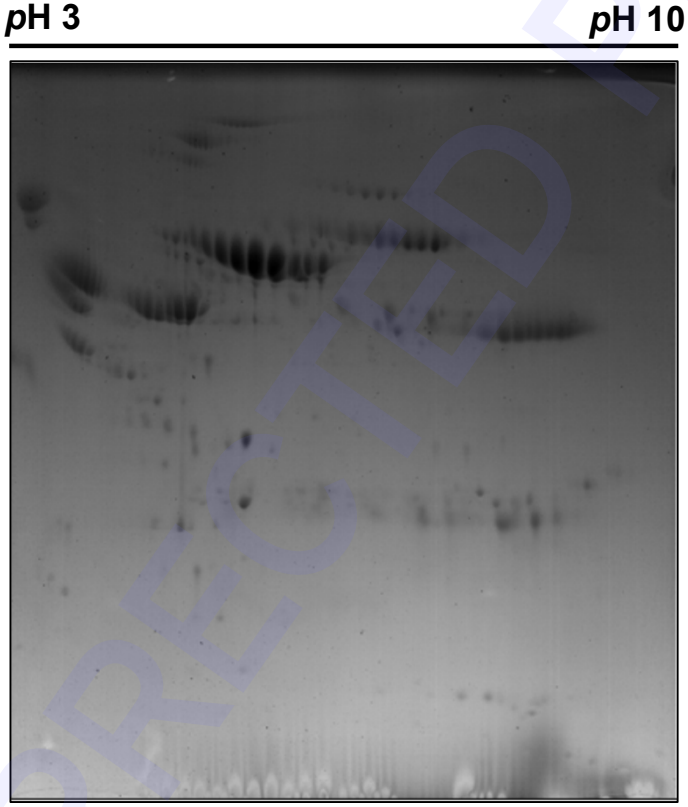
Fig. 6. Fig. 4A & B

A

a. C group



b. S group



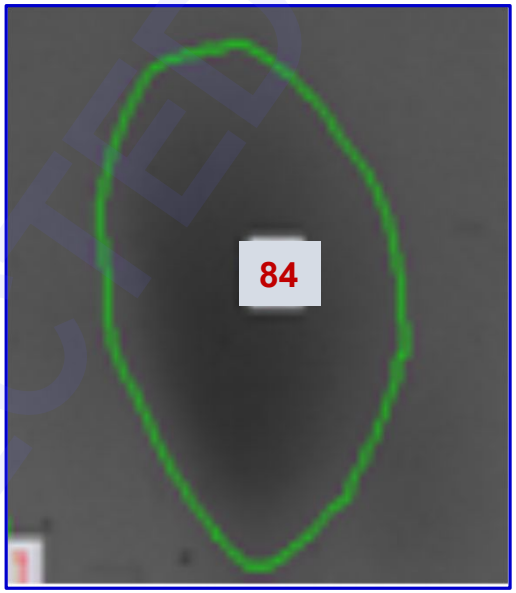
c. L group

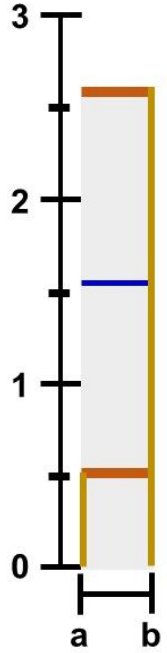
Supplementary Fig. 1 (Bahk et al.)

B

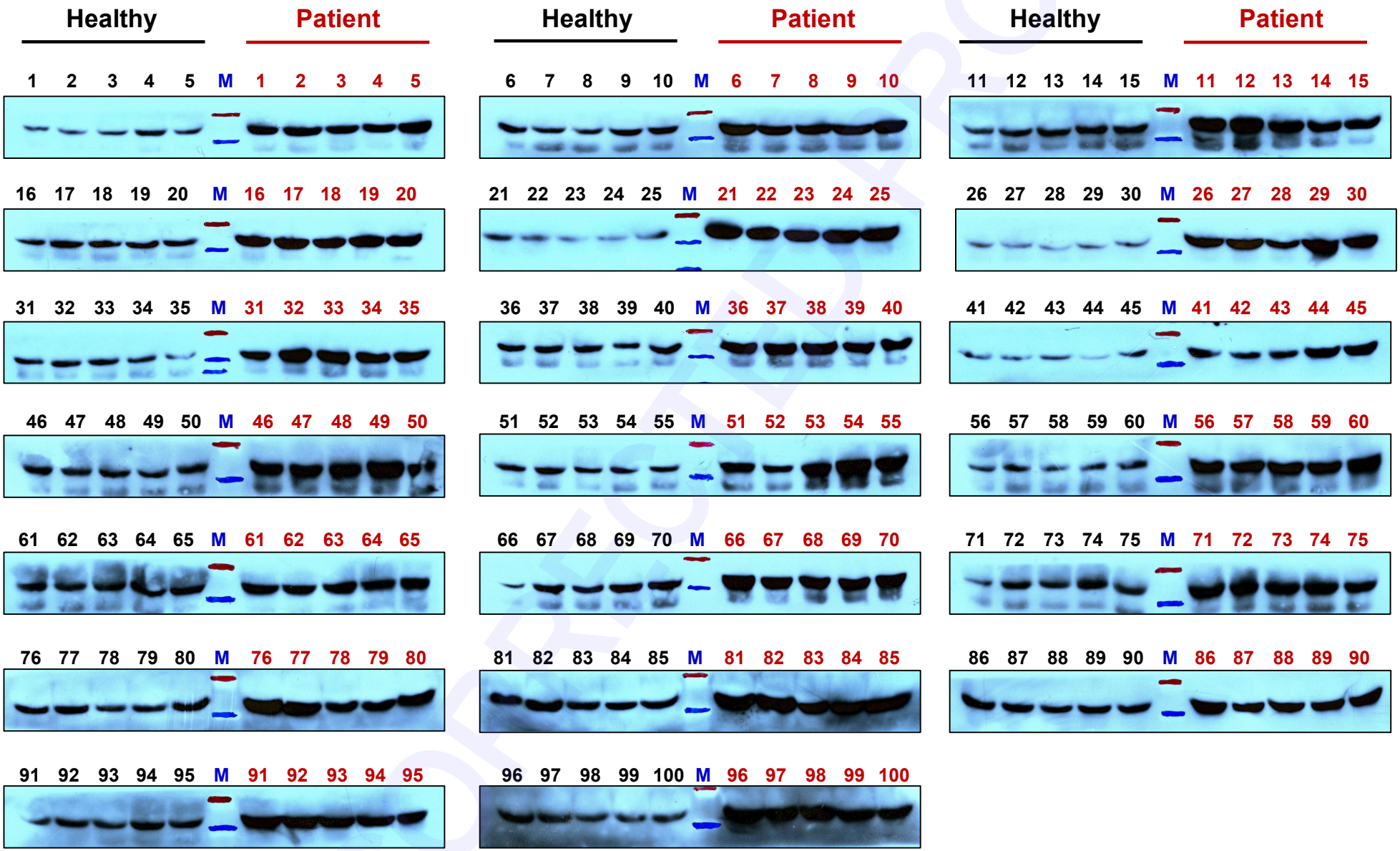
A. C group

B. S group





Supplementary Fig. 1 (Bahk et al.)



Supplementary Fig. 2 (Bahk et al.)

Supplementary Fig. 1. (A). Comparison of 2-DE maps of whole plasma from healthy donors (C group, a), donors for whom the outbreak time coincides with the mosquito's activity period (S group, b), and donors for whom the outbreak time does not coincide with the mosquito's activity period (L group, c). The gels were visualized by Coomassie brilliant blue G-250 staining. (B). Magnified regions of the differentially expressed α1-antichymotrypsin (AACT) protein spots on each gel between the healthy donors and the S group vivax malaria patients. Circled spots were identified as AACT proteins through analysis with 2-DE, SDS-PAGE and LC-MS/MS followed by identification with the program MASCOT.

Supplementary Fig. 2. AACT in the plasma of healthy donors and vivax malaria patients detected by chemiluminescence: black numbered lanes are healthy group (100 samples) and red numbered lanes (100 samples) are vivax malaria patients. For immunoblot analysis, equal amounts (30 μg) of whole plasma from individuals were analyzed with a commercially available anti-AACT monoclonal antibody followed by a goat anti-mouse secondary antibody coupled to horseradish peroxidase. The immunoreactive proteins on the NC membrane were detected by chemiluminescence on X-ray film.