FINAL DETAILED REPORT

The tasks planned in the application were successfully carried out in line with the objectives set out in the application.

OBJECTIVE N° 1. OBTAINING THE NUMBER OF BLOOD SAMPLES SUFFICIENT FOR PERFORMING IN VITRO TESTING AND FOR THE DEVELOPMENT OF A METHOD FOR MEASURING NET

Task 1.1 Continued expansion of our blood bank with blood samples (serum, EDTA-, and citrated plasma, stored at -80 °C) obtained from 80 C1-INH-HAE patients during attack-free periods.

Since the application was submitted, 21 new C1-INH-HAE patients have been diagnosed, and we are currently caring for 198 C1-INH-HAE patients, whose blood samples are available after diagnosis and during follow-up visits. We continue to collect serum, EDTA plasma, citrate plasma and DNA samples from all patients.

Task 1.2 Collection of blood samples from 80 sex- and age-matched controls to expand the available pool.

Since the application was submitted, we have collected serum, EDTA plasma and citrate plasma blood samples from a total of 217 healthy individuals.

Task 1.3 Collection of blood samples from C1-INH-HAE patients during at least 25 edematous attacks and symptom-free period from the same patients.

Since the application was submitted, blood samples have been taken from 139 patients with C1-INH-HAE during angioedema attacks and during asymptomatic periods.

Task 1.4 Development and calibration of a method for measuring NET.

We introduced a method in our laboratory to isolate neutrophil cells. After preparation, we stimulated the cells for NET formation. We collected the formed NET to use as a calibration standard for measuring NET. We used three commercial antibodies against NET-specific cell components (i.e. anti-MPO, anti-citrullinated H3 histone, and anti-dsDNA). We tested the specificity of the antibodies with immunoflourescent microscopy and simultaneously, we checked NET formation in the stimulated cells. We also tried these antibodies in an ELISA system, because we intend to develop an ELISA method for NET detection in plasma samples. The anti-MPO and the anti-dsDNA antibody both appeared to work well together. We continue the development of the ELISA method for measuring NET.
Further, we started complement testing in blood samples collected during the presence of erythema marginatum (EM). The following complement components were measured in blood samples of C1-INH-HAE patients during symptom-free periods, and during the occurrence of EM: C3, C4, C1q, CH50, C1-INH activity, C1-INH concentration, I-Factor, B-Factor, H-Factor, anti-H Factor antibodies, anti-C1q IgG antibodies, anti-C1-INH IgG, anti-C1-INH IgA, anti-C1-INH IgM antibodies, alternative pathway, MBL lectin-pathway. Compared with the remission period, our preliminary results showed a significant decrease of C3, C4 and anti-C1-

INH IgM antibody levels during the occurrence of EM.

OBJECTIVE N° 2. INVESTIGATION OF THE LABORATORY PARAMETERS OF NEUTROPHIL ACTIVATION IN THE BLOOD OF C1-INH-HAE PATIENTS AND OF HEALTHY INDIVIDUALS

Task 2.1. Determination of cytokines characterizing neutrophil activation – such as GM-CSF, IL-1β, CXCL-1 [chemokine (C-X-C motif) ligand 1] LTB4 (leukotriene B4) – and negative controls (monocyte chemoattractant protein-1 (MCP-1), chemokine (C-C motif) ligand 5 (CCL5) in plasma samples obtained from 80 C1-INH-HAE patients during attack-free periods and from 80 healthy controls, using commercial ELISA kits and in-house ELISA techniques.

Task 2.2. Measurement of NET in plasma samples obtained from 80 C1-INH-HAE patients during attack-free periods and from 80 healthy controls (using the in-house developed ELISA test).

We measured the levels of neutrophil-related PRNT3, TNFα, IL-8, NE, MPO, LTB4, using MCP-1 and CCL5 as negative control, with a commercial ELISA kits, as well as the levels of NET with our in-house ELISA (developed during the initial year of the project) in the samples obtained from 80 C1-INH-HAE patients during attack-free periods and from 80 healthy controls.

Task 2.3. Determination of cortisol and ACTH levels in plasma samples obtained from 80 C1-INH-HAE patients during attack-free periods and from 80 healthy controls, using electro-chemiluminescence immunoassay.

We measured cortisol and ACTH levels in 12 serum and 12 plasma samples collected from C1-INH-HAE patients during the characterization of the dynamics of a HAE attack, as well as obtained from healthy controls at matching time points. Peak ACTH level (which was 3 times compared to the baseline value) was detected in the hour immediately preceding the onset of the HAE attack. By the time edema formation began, ACTH level was returning to baseline and then, stayed low during the remaining part of the observation period. The highest cortisol level was measured in blood samples drawn during the initial stage of edema formation, with a one-hour difference compared with sampling for ACTH determination.
We developed a new method to measure the strength of the HUVEC/neutrophil association.

Task 2.4. Determination of cytokines levels characterizing neutrophil activation – GM-CSF, IL-1β, CXCL-1 and LTB4 (using commercial ELISA) – and negative controls (MCP-1, CCL5) in plasma samples obtained from 25 C1-INH-HAE patients (from a subgroup of 80 patients) during attack-free periods, and from the same 25 patients during edematous attacks.

We measured the levels of neutrophil-related PRNT3, TNFa, IL-8, NE, MPO, LTB4, using MCP-1 and CCL5 as a negative control with a commercial ELISA KIT with our in-house ELISA (developed during the initial year of the project), in the samples obtained from 14 C1-INH-HAE patients during HAE attacks and from 8 patients during EM (a subgroup of 77 patients), and from one C1-INH-HAE patient at different times, during follow-up of an HAE attack.

Task 2. 5. Determination of NET characterizing neutrophil activation (using the in-house developed ELISA test), in plasma samples obtained from 25 C1-INH-HAE patients (a subgroup of 80 patients) during attack-free periods, and from the same 25 patients during edematous attacks.

We measured the level of NET with our in-house ELISA (developed during the first years of the project), in the samples collected from 14 C1-INH-HAE patients during HAE attacks and from 8 patients during EM (a subgroup of 77 patients), and from one C1-INH-HAE patient at different times, during follow-up of a HAE attack.

Task 2.6. Determination of cortisol and ACTH levels that play a role in the neutrophil accumulation with electrochemiluminescence immunoassay in plasma samples, obtained from 25 C1-INH-HAE patients (from the subgroup of 80 patients) during attack-free periods, and from the same 25 patients during edematous attacks.

We collected 177 samples from symptom-free C1-INH-HAE patients (77), healthy controls (78), C1-INH-HAE patients during EM (8, from the subgroup of 77 patients) and during HAE attack (14, from the subgroup of 77 patients). ACTH and cortisol levels were determined from the samples. We could not find any difference between the ACTH and cortisol levels of patients and the of healthy controls.

Task 2.7. Determination of enzyme levels (neutrophil elastase, myeloperoxidase, proteinase 3), cytokine levels (IL-8, GM-CSF, IL-1β, CXCL-1 and LTB4 (using commercial ELISA), NET, cortisol and ACTH levels in plasma samples obtained from a C1-INH-HAE patient at different times, but during the follow-up of an edematous attack.

We measured the level of neutrophil-related PRNT3, TNFa, IL-8, NE, MPO, LTB4, using MCP-1 and CCL5 as a negative control with a commercial ELISA KIT with our in-house ELISA (developed during the initial year of the project), in the samples obtained from 14 C1-INH-HAE patients during HAE attacks and from 8 patients during EM (a subgroup of 77 patients), and from one C1-INH-HAE patient at different times, during follow-up of an HAE attack.

The results of Tasks 2.1, 2.2, 2.3 and 2.4 have been published in a manuscript in Clinical Reviews in Allergy & Immunology.

Kajdácsi E, Veszeli N, Mező B, Jandrasics Z, Kőhalmi KV, Ferrara AL, Cervenak L, Varga L, Farkas H. Pathways of Neutrophil Granulocyte Activation in Hereditary Angioedema with C1 Inhibitor Deficiency. Clin Rev Allergy Immunol. 2021 Jun;60(3):383-395. doi: 10.1007/s12016-021-08847-4. Epub 2021 Feb 19. PMID: 33606193; PMCID: PMC8272702.

OBJECTIVE N° 3. IN VITRO ACTIVATION OF NEUTROPHILS ISOLATED FROM C1-INH-HAE PATIENTS AND FROM HEALTHY CONTROLS

Task 3.1. Isolation of neutrophils from whole blood samples from 25 patients and 25 healthy controls during symptom-free periods, using dextran sedimentation, Ficoll-Paque gradient centrifugation, and osmotic lysis of any remaining red blood cells. The cells will be activated with LPS, histamine, bradykinin, and immobilized immune complexes, as well as

with the reference compound PMA (Phorbol myristate acetate). Functional readouts will include the measurement of ROS (reactive oxygen species) with Amplex Red fluorescent dye; of the calcium signal by Fura-2 fluorescence; as well as of PRTN3, NET, neutrophil elastase, and myeloperoxidase by the ELISA method.

Task 3.2. HUVEC will be cultured on 96-well plates. Fluorescent labeling (Oregon Green) of neutrophil granulocytes, isolated from 10 patients during a symptom-free period and from 10 healthy controls, will be performed. The labeled granulocytes will be added to the culture of endothelial cells to study adherence by fluorescent microscopy. The experiment will be repeated with HUVEC treated with pro-inflammatory activators (LPS, histamine, bradykinin),. The (LPS, histamine, and bradykinin) activated neutrophil cells, isolated from patients and healthy controls, will be added to the untreated endothelial cells to study leukocyte adherence.

Task 3.3. The experiments set out in Task 3.2 will be repeated in the presence/absence of C1-INH.

We finished the development of a new method that aims to measure the strength of the HUVEC/neutrophil bonding. We measured the adhesion of the neutrophil granulocytes (NGs) of the patients and healthy controls to HUVEC cells. Parallel with these measurements we always measured the calcium signal and ROS production of NGs which got different treatment and also collected supernatant of NGs treated with different agents.
We found that the basic ROS production was higher of the NGs from the patients compared to the ROS production of the NGs from the controls.
The BK and C1-INH had no effect on the adhesion of the NGs to HUVECs. The LPS and PMA treatment increased the NGs' adhesion to the HUVECs, but these increases were lower in the case of the patients compared to the controls.
Our study were completed by Fluorescence-activated Cell Sorting (FACS) measurements. FACS from whole blood samples taken from peripheral blood of 20 C1-INH-HAE patients and 21 age-matched healthy controls. After the red blood cells lysis from the whole blood, the mean fluorescence intensity (MFI) of the following markers were determined: CD11a, CD11b, CD13, CD16, CD33, CD44, CD45, CD49b, CD49d, CD49e, CD51, CD61, CD62p, CD63, CD66b, CD73, CD114, CD116, CD170, CD177, CD182, CD184, CD191, CD192, CD193, CD195
A significantly higher CD61 MFI was measured in healthy controls.

The results were presented at the 12th C1-Inhibitor Deficiency & Angioedema Workshop and at the 50th Meeting of the Hungarian Society of Immunology and the 50th Meeting of the Hungarian Society of Allergology and Clinical Immunology.

Erika Kajdácsi, Zsófia Pólai, Zsuzsanna Balla, László Cervenak, Henriette Farkas
Investigation of neutrophil granulocyte function in patients with hereditary angioedema
12th C1-inhibitor Deficiency & Angioedema Workshop, 2021. június 3-6., Virtual event

Kajdácsi Erika, Pólai Zsófia, Balla Zsuzsanna, Cervenak László, Farkas Henriette Neutrofil granulociták funkciójának vizsgálata herediter angioödémában; Magyar Immunológiai Társaság 50. Vándorgyűlése, 2021. október 20-22, Kecskemét

Kajdácsi Erika Neutrofil granulociták funkciójának vizsgálata herediter angioödémában
MAKIT 2022 - A Magyar Allergológiai és Klinikai Immunológiai Társaság 50. Kongresszusa, 2022. május 5-7.

OBJECTIVE N° 4. ANALYSIS OF THE DATA ACCUMULATED FROM STUDIES

Task 4. 1. The findings from serological and cell culturing studies will be compared. The results will be interpreted, in order to confirm our hypothesis and to answer the research questions. The results of the studies will be compared with the following: the demographical properties of the patients; observed clinical manifestations; the number, location, and severity of angioedematous attacks, trigger factors; treatment. The results of the analysis will be used to determine the role of neutrophils in C1-INH-HAE attacks. Study results will be presented and published.

We finished the measurement of the activation markers of NGs from the collected supernatant. We found that the NGs originated from the patients produced significantly higher MPO, ELA2 and NET than the NGs originated from the controls even after 4 h and after 24 h incubation with the stimulator treatments.

A manuscript summarising all the results obtained from the isolated NGs has been written. This will be published in the journal by the end of 2022.

During the research we also had the opportunity to analyze the clinical and laboratory data of the patients. The results have been published.

Zsuzsanna Balla, Noémi Andrási, Zsófia Pólai, Beáta Visy, Ibolya Czaller, György Temesszentandrási, Dorottya Csuka, Lilian Varga, Henriette Farkas: The characteristics of upper airway edema in hereditary and acquired angioedema with C1-inhibitor deficiency, Clin Transl Allergy, 2021 Dec;11(10):e12083 doi: 10.1002/clt2.12083..

Diagnosing Pediatric Patients With Hereditary C1-Inhibitor Deficiency—Experience From the Hungarian Angioedema Center of Reference and Excellence

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Overview of SERPING1 Variations Identified in Hungarian Patients With Hereditary Angioedema
Edina Szabó, Dorottya Csuka, Noémi Andrási, Lilian Varga, Henriette Farkas, Ágnes Szilágyi Front Allergy. 2022; 3: 836465. Published online 2022 Mar 17. doi: 10.3389/falgy.2022.836465 PMCID:PMC8974857

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