Increased thromboinflammatory load in hereditary angioedema

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List of abbreviations

Abbreviation	Definition
β-TG	β-thromboglobulin
B2R	bradykinin 2 receptor
C1Inh	C1 Inhibitor
CCL	chemokine (C-C motif) ligand
CXCL	chemokine (C-X-C motif) ligand
ELISA	enzyme-linked immunosorbent assay
F1+2	prothrombin fragment 1+2
FGF	fibroblast growth factor
FXI	factor XI
FXII	factor XII
FXIIa	activated factor XII
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HAE	hereditary angioedema
HMWK	high molecular weight kininogen
IFN	interferon

IL	interleukin
IL-1-RA	IL-1 receptor antagonist
mAb	monoclonal antibody
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
PAI-1	plasminogen activator inhibitor-1
PAR1	protease-activated receptor 1
PDGF	platelet-derived growth factor
PK	plasma kallikrein
SLE	systemic lupus erythematosus
TCC	terminal C5b-9 complement complex
TNF	tumour necrosis factor
VTE	venous thromboembolism

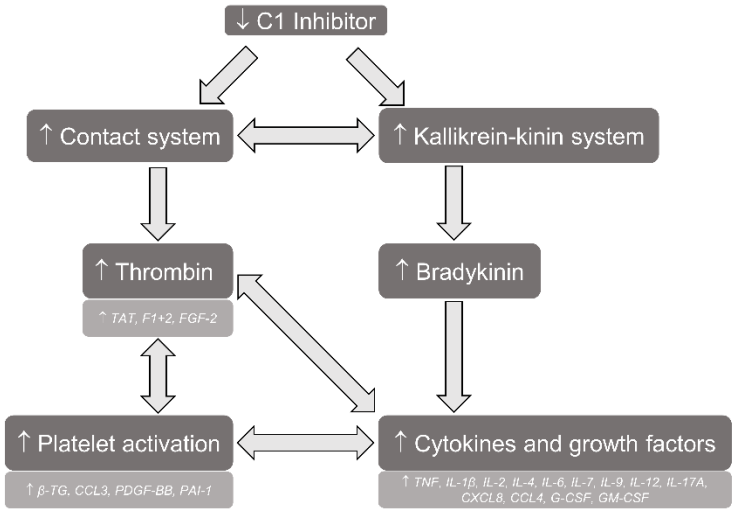
ABSTRACT

C1 inhibitor (C1Inh) is a serine protease inhibitor involved in the kallikrein-kinin system, the complement system, the coagulation system and the fibrinolytic system. In addition to the plasma leakage observed in hereditary angioedema (HAE), C1Inh deficiency may also affect these systems, which are important for thrombosis and inflammation. The aim of this study was to investigate the thromboinflammatory load in C1Inh deficiency. We measured 27 cytokines including interleukins, chemokines, interferons, growth factors and regulators using multiplex technology. Complement activation (C4d, C3bc and sC5b-C9/TCC), haemostatic markers (β-thromboglobulin (β-TG), thrombin-antithrombin complexes (TAT), prothrombin fragment 1+2 (F1+2), active plasminogen activator inhibitor-1 (PAI-1)) and the neutrophil activation marker myeloperoxidase (MPO) were measured by enzyme immunoassays. Plasma and serum samples were collected from 20 patients with HAE type 1 or 2 in clinical remission and compared with 20 healthy age- and sex-matched controls. Compared to healthy controls, HAE patients had significantly higher levels of tumour necrosis factor (TNF), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-7, IL-9, IL-12 and IL-17A, chemokine ligand (CXCL) 8, chemokine ligand (CCL) 3, CCL4, IL-1 receptor antagonist (IL-1RA), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF) 2 and platelet-derived growth factor (PDGF)-BB. HAE patients also had higher levels of TAT and F1+2. Although granulocyte colony-stimulating factor (G-CSF), β-TG and PAI-1 were higher in HAE patients, the differences did not reach statistical significance after correction for multiple testing. In conclusion, C1Inh deficiency is associated with an increased baseline thromboinflammatory load.

These findings may reflect that HAE patients are in a subclinical attack state outside of clinically apparent oedema attacks.

Keywords: Hereditary Angioedema; C1 Inhibitor; Bradykinin; Cytokines; Complement

Graphical abstract



Introduction:

Hereditary angioedema (HAE) is a rare genetic condition affecting approximately 1 in 50,000 people worldwide. HAE is characterised by spontaneous or traumatically induced angioedemas of skin and mucus membranes [1].

The underlying cause of this condition is a lack of functional C1 inhibitor (C1Inh) due to mutations in the SERPIN G1 gene, resulting in either a quantitative or qualitative deficit of C1Inh [2]. HAE type 1 refers to a quantitative deficit with low plasma C1Inh levels, whereas HAE type 2 refers to a qualitative deficit with dysfunctional C1Inh, even with high plasma concentrations [3]. Other types of HAE, in which the pathophysiology is not related to C1Inh deficiency, have also been identified [4].

C1Inh is the main regulator of the kallikrein-kinin system. Here, it inhibits plasma kallikrein (PK) [5], a serine protease that generates bradykinin through its proteolytic action on high molecular weight kininogen (HMWK) [6] (Fig. 1).

The kallikrein-kinin system is closely linked to the contact system, with PK playing a central role in both [7] (Fig. 1). The contact system refers to a proinflammatory and procoagulant system consisting of the serine proteases factor XII (FXII) and factor XI (FXI), in addition to PK [8]. Upon contact system activation, activated FXII (FXIIa) and PK reciprocally activate each other, creating a positive feedback loop between the contact and kallikrein-kinin systems [7] (Fig. 1). C1Inh inhibits all three proteases in the contact system [9,10], and its deficiency leads to uncontrolled contact system activation [11]. Contact system dysregulation is likely to be central to the HAE pathogenesis, as selective inhibition of FXIIa has recently been shown to be effective in preventing HAE attacks [12].

Inadequate regulation of the kallikrein-kinin and contact systems by C1Inh causes excessive production of the nonapeptide bradykinin which acts on endothelial bradykinin 2 receptors (B2R). B2R promote vascular permeability and their uncontrolled activation causes the swellings characteristic of HAE [13].

C1Inh also plays a role in the classical and lectin complement pathways [14,15], and was indeed named for its inhibitory effect on complement factors C1r and C1s [16]. C1Inh thus acts as a regulator of the autocatalytic activation of C1qrs. In C1Inh deficiency, this autocatalysis of C1 proceeds largely uninhibited [17], leading to downstream consumption of complement factors C2 and C4 [18]. In contrast, C3 levels are within the normal range [19], and activation of C3 and the terminal C5b-9 (TCC) pathway are modestly or not increased [20,21].

Furthermore, C1Inh inhibits plasmin and tissue plasminogen activator in the fibrinolytic system and thrombin in the coagulation system [22–24].

Bradykinin, in addition to its vasoactive effects, also has pro-inflammatory properties, promoting the upregulation of cytokines such as TNF, IL-1β, IL-2, IL-6, IL-10, CCL2 and CXCL8 (IL-8) [25]. Bradykinin has also been shown to upregulate growth factors such as G-CSF and GM-CSF *in vitro* [26]. While bradykinin itself is impractical to measure directly due to its short plasma half-life [27], measurements of cleaved bradykinin precursors or metabolites (bradykinin 1-5) suggest that HAE patients have elevated bradykinin levels also in clinical remission [28,29].

Although C1Inh deficiency affects physiological systems important for inflammation and haemostasis, previous studies of HAE patients in clinical remission have mainly

investigated inflammatory markers or markers of haemostasis separately. In this study, we therefore conduct a comprehensive investigation of both inflammatory and haemostatic markers in order to assess the baseline systemic thromboinflammatory load in HAE patients with C1Inh deficiency.

Materials and methods:

Patients:

The diagnosis of HAE type 1 or 2 was based on low antigenic or functional C1Inh in samples obtained either for diagnostic purposes in angioedema patients, or for screening purposes in individuals with a parent with known HAE. A total of 20 patients were included, of whom 13 patients had HAE type 1, and 7 patients had HAE type 2. To be included, the patients had to be in clinical remission and not have used C1Inh concentrate for at least 48h prior to sampling. None of the patients were using prophylaxis other than C1Inh concentrate. As controls, we used plasma from 20 healthy volunteers matched for sex and age (+/- 10 years). There were 9 males and 11 females in both groups. The mean age of the patients was 45.9 years (range 24 – 65), the mean age of the controls was 46.3 years (range 27 – 67).

Patient and control samples were collected with informed written consent between January 2019 and May 2020. The study has been approved by the Regional Ethical Committee of South-East Norway (ref.no. 2018/1289/REK Sør-Øst C).

Sample collection:

All samples were collected and stored according to our strict laboratory protocol based on our previous published methods for optimal sampling for complement activation products and cytokines [30,31]. Briefly, blood collected in citrate and EDTA

vials was stored on ice immediately after collection and centrifuged within 10 minutes at 2500 x *g* for 15 minutes at 4ºC. Serum samples were stored in room temperature for 30 minutes before centrifugation at 2500 x *g* for 15 minutes at 4ºC. Plasma and serum were then immediately aliquoted and stored at -70ºC.

Enzyme-linked immunosorbent assays (ELISAs)

Complement activation products:

Soluble terminal C5b-9 complement complex (TCC), detected by the monoclonal antibody (mAb) aE11 which recognizes a neoepitope in activated C9, and C3bc, detected by the mAb bH6 which recognizes a common neoepitope in C3b, iC3b and C3c, were measured in EDTA plasma using in-house ELISA-kits as previously described [32]. C4 activation product C4d was measured using a commercially available ELISA kit (COMPL C4d RUO, Svar Life Sciences, Malmö, Sweden) according to the manufacturer's instructions of use.

Haemostasis:

Platelet β-thromboglobulin (β-TG) was measured in EDTA plasma diluted 1:250, using a commercial ELISA kit (Human CXCL7/NAP-2 DuoSet ELISA, R&D Systems, Minneapolis, MO) according to the manufacturer's instructions of use.

Prothrombin fragment 1+2 (F1+2) were measured in citrated plasma using a commercial ELISA kit from Siemens (Enzygnost F1+2 monoclonal, Siemens Healthineers, Erlangen, Germany). The experiments were performed according to the manufacturer's instructions of use. In 7 out of 20 HAE patients, the readings were above

the upper calculation limit. These values were set to the highest standard concentration plus 1.

Thrombin-antithrombin complexes (TAT) were measured in citrated plasma using a commercial ELISA kit (Enzygnost TAT Micro, Siemens Healthineers, Erlangen, Germany) according to the manufacturer's instructions of use.

Active plasminogen activator inhibitor-1 (PAI-1) was measured in citrated plasma using a commercial ELISA kit (IHUPAI1KT, Molecular Innovations, Novi, MI) according to the manufacturer's instructions of use.

Neutrophil activation:

Myeloperoxidase (MPO) was measured in serum diluted 1:500 using a commercial ELISA kit (Human Myeloperoxidase DuoSet ELISA, R&D Systems, Minneapolis, MO) according to the manufacturer's instructions of use.

Multiplex cytokine immunoassay:

Cytokines, including interleukins, chemokines, interferons, growth factors and regulators were measured in EDTA plasma using a commercially available multiplex cytokine immunoassay (Bio-Plex Pro Human Cytokine 27-plex Assay, Bio-Rad Laboratories, Hercules, CA) as specified in the manufacturer's instructions of use. Values below the detection threshold were included as random numbers between 0.01 and the lowest standard or extrapolated value or using the RANDBETWEEN function in Microsoft Excel 2016 version 2209. All measurements of IL-15 and human VEGF were

below the detection limit, and were therefore excluded from the analysis, as was RANTES, as this chemokine is released from platelets *in vitro* after sampling.

Statistical analysis:

Statistical analyses were performed using GraphPad Prism version 9.4.1 (681) from GraphPad Software, LLC. Only the data for IL-4, IL-7, G-CSF, CCL2 (MCP-1) and TNF were normally distributed as determined by the Shapiro-Wilks test, and p-values for these variables were therefore calculated using a two-tailed unpaired t-test for the main analyses All other p-values were determined using a two-tailed Mann-Whitney test, including the subgroup analyses. Holm-Bonferroni sequential correction was then done to correct for multiple testing [33,34]. Both uncorrected and corrected p-values are reported. Statistical significance was defined as a p-value < 0.05.

Results:

Complement activation products:

HAE patients had significantly higher C4d plasma concentrations than the healthy controls (Fig. 2, left panel), as expected. C3bc, cleavage fragments from the activation of C3, were also found to be significantly elevated in HAE patients (Fig. 2, middle panel). TCC levels were similar between HAE patients in remission and controls (Fig. 2, right panel).

Haemostasis:

Platelet β-thromboglobulin (β-TG), a marker of platelet activation, was higher in the HAE group. After correction for multiple testing, the difference from the control group did not reach statistical significance (uncorrected p = 0.0095, corrected p = 0.105) (Fig. 3, left panel).

F1+2 and TAT, both markers of coagulation, were significantly elevated in HAE patients compared to controls (Fig. 3, middle two panels).

PAI-1, a serine protease inhibitor that regulates the fibrinolytic system, was higher in the HAE group than in the controls. The difference did not reach statistical significance after correction for multiple testing (uncorrected p = 0.0042, corrected p = 0.0504) (Fig. 3, right panel).

Neutrophils:

MPO, the most abundant protein in neutrophils and a biochemical marker of neutrophil activation, was present at similar levels in HAE patients and controls (Fig. 4).

Cytokines I - Interleukins:

Tumour necrosis factor (TNF), interleukin- (IL)-1β, IL-2, IL-4, IL-6, IL-7, IL-9, IL-12 and IL-17A were all found at significantly higher plasma levels in HAE patients compared to the controls (Fig. 5). Levels of IL-5, IL-10 and IL-13 were not significantly different between the groups (Fig. 5).

Cytokines II - Chemokines:

Chemokine ligand (CXCL) 8 (IL-8), chemokine ligand (CCL) 3 (macrophage inflammatory protein (MIP)-1α) and CCL4 (MIP-1β) were significantly elevated in the HAE group compared to the control group (Fig. 6, upper left, lower middle and right panels). Levels of eotaxin, CXCL10 and CCL2 were not significantly different between the groups (Fig. 6, upper middle and right and lower left panels).

Cytokines III: regulators, growth factors and interferons:

IL-1 receptor antagonist (IL-1RA), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF) 2 and platelet-derived growth factor (PDGF)-BB were significantly higher in the HAE patients than in the controls (Fig. 7, upper left, lower middle, upper and lower right panels). Granulocyte colony-stimulating factor (G-CSF) was higher in the HAE group. After correction for multiple testing, the difference did not reach statistical significance (uncorrected p = 0.0248, corrected p = 0.248) (Fig. 7, lower left panel). Interferon- (IFN-) γ, did not differ between the groups (Fig. 7, upper middle panel).

Subgroup analyses:

HAE type 2 patients had higher levels of MPO, IL-2, IL-4, eotaxin and CCL4 compared to HAE type 1 patients. Male HAE patients had higher levels of IL-5, IL-13, CM-CSF and CCL2 compared to female patients. When comparing patients on and off prophylaxis (defined as having received C1Inh concentrate within one week prior to

sampling or not), IL-5 was higher in patients on prophylaxis. There were no significant differences within the subgroups after Holm-Bonferroni correction (Fig. 8).

Discussion:

Even in the absence of clinically evident oedema, our study shows that HAE-C1Inh is associated with an increased thromboinflammatory load, as there is evidence for simultaneous hypercoagulation and low-grade inflammation.

Although HAE patients are biochemically in a hypercoagulable state, a clear association between HAE and venous thromboembolism (VTE) has not been established. A recent retrospective registry-based cohort study of Swedish patients found that HAE patients had a significantly higher risk of having registered VTE-related ICD-10 codes compared to controls [35,36]. However, these findings are in contrast to previous studies which found no association between thromboembolism and HAE, even in the presence of elevated D-dimer [37].

An increased risk of autoimmune diseases, particularly systemic lupus erythematosus (SLE), has been reported in HAE patients [38]. While increased levels of inflammatory cytokines such as IL-6 and IL-17 may play a role, the most important factor in SLE risk in HAE patients is likely to be the acquired C2 and C4 deficiency resulting from uncontrolled C1 autocatalysis. The probable protective mechanism of C2 and C4 is their role in promoting the clearance of immune complexes and apoptotic debris that may serve as autoantigens [39], and their deficiency may therefore predispose to SLE.

We found higher levels of F1+2, a marker of prothrombin activation and thrombin generation, and TAT, a marker of thrombin neutralisation, in the HAE group. This is consistent with findings of Csuka et al. [40]. The increased thrombin generation in these patients may be linked to the increased contact system activation in C1Inh deficiency [35]. Also, cytokines like IL-6, TNF and IL-1β, all of which are increased in HAE, may upregulate tissue factor and increase thrombin generation via the extrinsic coagulation system [41]. C1Inh infusion in HAE patients with elevated basal F1+2 has also been shown to reduce F1+2 levels [42], demonstrating the relationship between C1Inh and thrombin generation in HAE.

We found higher levels of PAI-1 in HAE patients than in controls, although this did not reach statistical significance after correction for multiple testing. Elevated levels of PAI-1 in HAE patients have been reported by Joseph et al. [43]. In contrast, the study by Csuka et al. [40], did not show significant differences in PAI-1 levels in HAE patients in remission as compared to controls, whereas they found PAI-1 to markedly decrease during HAE attacks in most of the patients. PAI-1 is an important inhibitor of the fibrinolytic system [44], and elevated levels could therefore contribute to a procoagulant state together with the activation of the coagulation cascade. However, PAI-1 alone does not reflect the status of the fibrinolytic system. We have previously shown that plasmin-alpha-2-antiplasmin complexes were normal in HAE patients in remission, but increased substantially during attacks [21]. Taken together, these data suggest that the role of PAI-1 in balancing coagulation and fibrinolysis in HAE in remission should be interpreted with caution.

β-TG, a protein abundant in platelet α-granules and a specific marker of platelet activation [45], was higher in the HAE population, although the difference did not reach statistical significance after Holm-Bonferroni correction. PDGF-BB, another protein released by platelets upon their activation, was also significantly higher in the HAE patients. Although PDGF-BB is a less specific marker of platelet activation as it can also be released by other cell types [46], together these findings may indicate increased platelet activation in HAE patients in remission. Upregulation of B2R by PDGF has been demonstrated *in vitro* [47], however, few studies on PDGF and HAE exist, and a possible role of PDGF in HAE has not been studied.

Thrombin is a potent platelet activator mainly through protease-activated receptor 1 (PAR1) [48], and increased thrombin generation may be a possible explanation for increased platelet activation in HAE patients. Increased levels of cytokines such as IL-1β and IL-12 could also contribute to platelet activation [49,50]. It is less likely that increased platelet activation in these patients is due to elevated levels of bradykinin, as bradykinin and its metabolite bradykinin 1-5 have been reported to inhibit rather than activate platelets [51,52].

Increased C3 activation was previously described by us [20], and confirmed in the present study. This C3 activation is, however, minor and unlikely to be of clinical significance, particularly as terminal pathway activation as measured by TCC, was not observed. C5 cleavage leads to the formation of C5a and TCC in equimolar amounts, indicating that HAE patients do not have elevated levels of the highly potent anaphylatoxin C5a when in remission.

We found that a substantial number of the measured cytokines were significantly elevated in the HAE patients in remission compared to controls, i.e. nine out of 12 interleukins, three out of six chemokines and four out of four growth factors, confirming some single observations in earlier studies [53–55]. The present study includes a more comprehensive panel of cytokines studied in HAE patients in remission, and the broad-spectrum increase indicates a substantial inflammatory load. The mechanism of cytokine upregulation is likely to be multifactorial. Bradykinin itself can stimulate the release of a number of cytokines including TNF [56], IL-1, IL-2 [57] IL-6, CXCL8 [58], G-CSF, GM-CSF and CCL2 [26]. In an experimental animal model in pigs, bradykinin infusion led to a significant increase in TNF and IL-6 [59]. Also, thrombin [60], and by extension fibrin [61], are known inducers of inflammatory cytokines. Furthermore, several cytokines can themselves upregulate the release of other cytokines [62].

Clearly, there are significant interactions between the physiological systems involved, and the broad specificity of C1Inh makes it likely that pathology may occur simultaneously at multiple sites, increasing the complexity of these interactions. A limitation of this observational study is thus that the direct causal mechanisms underlying the biochemical pathologies in HAE remain unclear.

We consider our selection of patients and controls selection to be an important strength of the study. Female sex and oestrogen are positively associated with HAE severity [63], and matching patients and controls by sex was therefore important.

Although age is not known to affect HAE severity [64], ageing is associated with changes in the coagulation and fibrinolytic systems and an increase in inflammatory markers [65,66], which was a strong rationale for also matching the patients and controls by age.

PK and FXIIa are targets for novel and likely future HAE therapies. The anti-PK monoclonal antibody (mAb) lanadelumab is already approved for patients in Europe and USA [67,68], and the anti-FXIIa mAb garadacimab has undergone phase 3 clinical trials with promising results [12]. The antisense nucleotide donidalorsen which inhibits hepatocyte production of prekallikrein has also shown promise in phase 2 clinical trials where the drug was administered every 4 weeks [69].

Comprehensive *in vivo* studies of thromboinflammation in HAE patients receiving highly specific prophylactic therapies may provide further insight into the pathophysiology underlying the biochemical abnormalities described in this paper.

As previous studies have shown significant changes in inflammatory and haemostatic parameters during acute swelling attacks compared to remission [70], we hypothesise that our findings reflect a subclinical HAE attack state characterised by low-grade thromboinflammation.

The above-mentioned mAbs, as well as donidalorsen, have much longer half-lives, and therefore the ability to maintain remission for longer, than other therapies currently on the market for HAE prophylaxis [71,72]. Assessment of thromboinflammatory markers in patients using such therapies may therefore be helpful in supporting or rejecting our hypothesis. Indeed, Fijen et al. studied coagulation and fibrinolysis markers in patients before and during treatment with donidalorsen, and their findings suggest a decrease in F1+2 as well as TAT complexes and D-dimer [69].

If patients in clinical remission are indeed in a subclinical HAE attack state, and furthermore can achieve biochemical remission on effective prophylactic therapy, markers of thromboinflammation may become valuable tools in the future to guide and individualise the choice and dosing of prophylactic HAE treatment.

We acknowledge that our study has a number of limitations. First, the ability to detect longitudinal changes in the thromboinflammatory state of HAE patients is limited by the cross-sectional design of our study. Repeated measurements over time would have provided valuable information on the stability of the observed thromboinflammatory pattern. Such longitudinal assessments may also help to identify the most representative markers of thromboinflammation in HAE.

Another limitation is the lack of confirmatory assays for specific markers such as IL-6 and TNF. Unfortunately, due to limited sample availability, we were unable to perform these additional tests, which would have helped to further validate and characterise the thromboinflammatory state in HAE patients.

It is also important to recognise the potential impact of the correction for multiple testing, despite the strong correlation between the uncorrected and corrected analyses in terms of statistical probability. Although we have applied correction methods to reduce the likelihood of type 1 errors, it is worth noting that such corrections may inadvertently increase the risk of type 2 errors. Given the interconnected and interdependent nature of the markers that were analysed, it is possible that some significant associations may have been missed [73]. To address this concern and to allow for a comprehensive assessment of statistical significance, we have included both uncorrected and corrected p-values in Figs. 2 to 8.

Finally, the numbers of patients in each subgroup in the subgroup analyses, including comparisons between HAE type 1 and 2, male and female patients, and patients on and off prophylaxis (defined as having received C1Inh concentrate within one week before sampling or not), were very small. Therefore, the results of the subgroup analyses should be interpreted with caution.

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The data underlying this article will be shared on reasonable request to the corresponding author.

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Permission to reproduce material from other sources:

No material from other sources was reproduced.

Clinical trial registration:

Not applicable to this study.

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Camilla Schjalm: Sample collection, Analyses, Interpretation, Writing - Review & Editing.

Erik Waage Nielsen: Conceptualization, Methodology, Supervision, Writing - Review & Editing

Tom Eirik Mollnes: Conceptualization, Methodology, Resources, Writing - Review & Editing, Visualization, Supervision

Authorship:

All authors have contributed substantially to the conception of the study and its design and/or the acquisition and analysis of data. All authors have edited the manuscript for important intellectual content. All authors have seen, reviewed and approved the final version of the manuscript and accept their accountability towards its accuracy.

References

1. Maurer M, Magerl M, Betschel S *et al.* The international WAO/EAACI guideline for the management of hereditary angioedema-The 2021 revision and update. *Allergy* 2022;**77**:1961–90.

2. Santacroce R, D'Andrea G, Maffione AB *et al.* The Genetics of Hereditary Angioedema: A Review. *J Clin Med* 2021;**10**:2023.

3. Rosen FS, Charache P, Pensky J *et al.* Hereditary Angioneurotic Edema: Two Genetic Variants. *Science* 1965;**148**:957–8.

4. Bork K, Wulff K, Möhl BS *et al.* Novel hereditary angioedema linked with a heparan sulfate 3-O-sulfotransferase 6 gene mutation. *J Allergy Clin Immunol* 2021;**148**:1041–8.

5. Schapira M, Scott CF, Colman RW. Contribution of Plasma Protease Inhibitors to the Inactivation of Kallikrein in Plasma. *J Clin Invest* 1982;**69**:462–8.

6. Thompson RE, Mandle R, Kaplan AP. Characterization of human high molecular weight kininogen. Procoagulant activity associated with the light chain of kinin-free high molecular weight kininogen. *J Exp Med* 1978;**147**:488–99.

7. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost* 2016;**14**:28–39.

8. Weidmann H, Heikaus L, Long AT *et al.* The plasma contact system, a protease cascade at the nexus of inflammation, coagulation and immunity. *Biochim Biophys Acta BBA - Mol Cell Res* 2017;**1864**:2118–27.

9. de Agostini A, Lijnen HR, Pixley RA *et al.* Inactivation of factor XII active fragment in normal plasma. Predominant role of C-1-inhibitor. *J Clin Invest* 1984;**73**:1542–9.

10. Wuillemin WA, Minnema M, Meijers JCM *et al.* Inactivation of Factor XIa in Human Plasma Assessed by Measuring Factor XIa-Protease Inhibitor Complexes: Major Role for C1-Inhibitor. *Blood* 1995;**85**:1517–26.

11. De Maat S, Hofman ZLM, Maas C. Hereditary angioedema: the plasma contact system out of control. *J Thromb Haemost* 2018;**16**:1674–85.

12. Craig TJ, Reshef A, Li HH *et al.* Efficacy and safety of garadacimab, a factor XIIa inhibitor for hereditary angioedema prevention (VANGUARD): a global, multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *The Lancet* 2023;**401**:1079–90.

13. Bouillet L, Mannic T, Arboleas M *et al.* Hereditary angioedema: Key role for kallikrein and bradykinin in vascular endothelial-cadherin cleavage and edema formation. *J Allergy Clin Immunol* 2011;**128**:232–4.

14. Ziccardi RJ. Activation of the early components of the classical complement pathway under physiologic conditions. *J Immunol* 1981;**126**:1769–73.

15. Presanis JS, Hajela K, Ambrus G *et al.* Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. *Mol Immunol* 2004;**40**:921–9.

16. Pensky J, Levy LR, Lepow IH. Partial Purification of a Serum Inhibitor of C'1-Esterase. *J Biol Chem* 1961;**236**:1674–9.

17. Bianchino AC, Poon PH, Schumaker VN. A mechanism for the spontaneous activation of the first component of complement, C1, and its regulation by C1-inhibitor. *J Immunol* 1988;**141**:3930–6.

18. Donaldson VH, Rosen FS. Action of Complement in Hereditary Angioneurotic Edema: The Role of C′1-Esterase *. *J Clin Invest* 1964;**43**:2204–13.

19. Brasher GW. Complement Component Analysis in Angioedema: Diagnostic Value. *Arch Dermatol* 1975;**111**:1140.

20. Nielsen EW, Johansen HT, Gaudesen Ø *et al.* C3 is Activated in Hereditary Angioedema, and C1/C1-Inhibitor Complexes Rise During Physical Stress in Untreated Patients. *Scand J Immunol* 1995;**42**:679–85.

21. Nielsen EW, Thidemann Johansen H, Høgäsen K *et al.* Activation of the Complement, Coagulation, Fibrinolytic and Kallikrein–Kinin Systems During Attacks of Hereditary Angioedema. *Scand J Immunol* 1996;**44**:185–92.

22. Harpel PC, Cooper NR. Studies on human plasma C1 inactivator-enzyme interactions. I. Mechanisms of interaction with C1s, plasmin, and trypsin. *J Clin Invest* 1975;**55**:593–604.

23. Booth NA, Walker E, Maughan R *et al.* Plasminogen Activator in Normal Subjects After Exercise and Venous Occlusion: t-PA Circulates as Complexes With Cl-Inhibitor and PAI-1. *Blood* 1987;**69**:1600–4.

24. Cugno M, Bos I, Lubbers Y *et al.* In vitro interaction of C1-inhibitor with thrombin. *Blood Coagul Fibrinolysis* 2001;**12**:253–60.

25. Rex DAB, Deepak K, Vaid N *et al.* A modular map of Bradykinin-mediated inflammatory signaling network. *J Cell Commun Signal* 2022;**16**:301–10.

26. Koyama S, Sato E, Numanami H *et al.* Bradykinin Stimulates Lung Fibroblasts to Release Neutrophil and Monocyte Chemotactic Activity. *Am J Respir Cell Mol Biol* 2000;**22**:75–84.

27. Cyr M, Lepage Y, Charles Blais J *et al.* Bradykinin and des-Arg9-bradykinin metabolic pathways and kinetics of activation of human plasma. *Am J Physiol-Heart Circ Physiol* 2001, DOI: 10.1152/ajpheart.2001.281.1.H275.

28. Hofman ZLM, de Maat S, Suffritti C *et al.* Cleaved kininogen as a biomarker for bradykinin release in hereditary angioedema. *J Allergy Clin Immunol* 2017;**140**:1700-1703.e8.

29. Seip KF, Bjerknes KC, Johansen HT *et al.* Bradykinin analysis revived – A validated method for determination of its stable metabolite in whole blood by LC–MS/MS. *J Chromatogr B* 2014;**947–948**:139–44.

30. Mollnes TE, Garred P, Bergseth G. Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation. *Clin Exp Immunol* 1988;**73**:484–8.

31. Hennø LT, Storjord E, Christiansen D *et al.* Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines - Consequences for defining reference values in healthy humans. *Cytokine* 2017;**97**:86–95.

32. Bergseth G, Ludviksen JK, Kirschfink M *et al.* An international serum standard for application in assays to detect human complement activation products. *Mol Immunol* 2013;**56**:232–9.

33. Gaetano J. Holm-Bonferroni sequential correction: An Excel calculator (1.3). 2018, DOI: 10.13140/RG.2.2.28346.49604.

34. Holm S. A Simple Sequentially Rejective Multiple Test Procedure. *Scand J Stat* 1979;**6**:65–70.

35. Grover SP, Sundler Björkman L, Egesten A *et al.* Hereditary angioedema is associated with an increased risk of venous thromboembolism. *J Thromb Haemost* 2022;**20**:2703–6.

36. Sundler Björkman L, Persson B, Aronsson D *et al.* Comorbidities in hereditary angioedema—A population-based cohort study. *Clin Transl Allergy* 2022;**12**:e12135.

37. Reshef A, Zanichelli A, Longhurst H *et al.* Elevated D-dimers in attacks of hereditary angioedema are not associated with increased thrombotic risk. *Allergy* 2015;**70**:506–13.

38. Tachdjian R, Borra S, Shrestha S *et al.* Burden of Autoimmune Disorders Among Patients With Hereditary Angioedema in the United States. *J Allergy Clin Immunol* 2020;**145**:AB102.

39. Elkon KB, Santer DM. Complement, interferon and lupus. *Curr Opin Immunol* 2012;**24**:665–70.

40. Csuka D, Veszeli N, Imreh É *et al.* Comprehensive study into the activation of the plasma enzyme systems during attacks of hereditary angioedema due to C1-inhibitor deficiency. *Orphanet J Rare Dis* 2015;**10**:132.

41. Iannucci J, Renehan W, Grammas P. Thrombin, a Mediator of Coagulation, Inflammation, and Neurotoxicity at the Neurovascular Interface: Implications for Alzheimer's Disease. *Front Neurosci* 2020;**14**:762.

42. Coppola L, Guastafierro S, Verrazzo G *et al.* C1 Inhibitor Infusion Modifies Platelet Activity in Hereditary Angioedema Patients. *Arch Pathol Lab Med* 2002;**126**:842–5.

43. Joseph K, Tholanikunnel BG, Wolf B *et al.* Deficiency of plasminogen activator inhibitor 2 in plasma of patients with hereditary angioedema with normal C1 inhibitor levels. *J Allergy Clin Immunol* 2016;**137**:1822-1829.e1.

44. Morrow GB, Mutch NJ. Past, Present, and Future Perspectives of Plasminogen Activator Inhibitor 1 (PAI-1). *Semin Thromb Hemost* 2022, DOI: 10.1055/s-0042-1758791.

45. Kaplan KL, Owen J. Plasma levels of beta-thromboglobulin and platelet factor 4 as indices of platelet activation in vivo. *Blood* 1981;**57**:199–202.

46. Demoulin J-B, Essaghir A. PDGF receptor signaling networks in normal and cancer cells. *Cytokine Growth Factor Rev* 2014;**25**:273–83.

47. Dixon BS, Sharma RV, Dennis MJ. The Bradykinin B2 Receptor Is a Delayed Early Response Gene for Platelet-derived Growth Factor in Arterial Smooth Muscle Cells *. *J Biol Chem* 1996;**271**:13324–32.

48. Andersen H, Greenberg DL, Fujikawa K *et al.* Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proc Natl Acad Sci U S A* 1999;**96**:11189–93.

49. Brown GT, Narayanan P, Li W *et al.* Lipopolysaccharide stimulates platelets through an IL-1β autocrine loop. *J Immunol Baltim Md 1950* 2013;**191**:10.4049/jimmunol.1300354.

50. Page MJ, Bester J, Pretorius E. Interleukin-12 and its procoagulant effect on erythrocytes, platelets and fibrin(ogen): the lesser known side of inflammation. *Br J Haematol* 2018;**180**:110–7.

51. Hasan AAK, Amenta S, Schmaier AH. Bradykinin and Its Metabolite, Arg-Pro-Pro-Gly-Phe, Are Selective Inhibitors of α-Thrombin–Induced Platelet Activation. *Circulation* 1996;**94**:517–28.

52. Prieto AR, Ma H, Huang R *et al.* Thrombostatin, a bradykinin metabolite, reduces platelet activation in a model of arterial wall injury. *Cardiovasc Res* 2002;**53**:984–92.

53. Arcoleo F, Salemi M, Porta AL *et al.* Upregulation of cytokines and IL-17 in patients with hereditary angioedema. *Clin Chem Lab Med* 2014;**52**:e91–3.

54. Arcoleo F, Lo Pizzo M, Misiano G *et al.* The complex alteration in the network of IL-17-type cytokines in patients with hereditary angioedema. *Clin Exp Med* 2018;**18**:355–61.

55. Kajdácsi E, Veszeli N, Mező B *et al.* Pathways of Neutrophil Granulocyte Activation in Hereditary Angioedema with C1 Inhibitor Deficiency. *Clin Rev Allergy Immunol* 2021, DOI: 10.1007/s12016-021-08847-4.

56. Tiffany CW, Burch RM. Bradykinin stimulates tumor necrosis factor and interleukin-1 release from macrophages. *FEBS Lett* 1989;**247**:189–92.

57. Paegelow I, Werner H, Vietinghoff G *et al.* Release of cytokines from isolated lung strips by bradykinin. *Inflamm Res* 1995;**44**:306–11.

58. Hayashi R, Yamashita N, Matsui S *et al.* Bradykinin stimulates IL-6 and IL-8 production by human lung fibroblasts through ERK- and p38 MAPK-dependent mechanisms. *Eur Respir J* 2000;**16**:452–8.

59. Seip KF, Evjenth B, Hovland A *et al.* Bradykinin-Induced Shock Increase Exhaled Nitric Oxide, Complement Activation and Cytokine Production in Pigs. *J Cardiol Clin Res* 2016;**4**:1057.

60. Kranzhöfer R, Clinton SK, Ishii K *et al.* Thrombin Potently Stimulates Cytokine Production in Human Vascular Smooth Muscle Cells but Not in Mononuclear Phagocytes. *Circ Res* 1996;**79**:286–94.

61. Jensen T, Kierulf P, Sandset PM *et al.* Fibrinogen and fibrin induce synthesis of proinflammatory cytokines from isolated peripheral blood mononuclear cells. *Thromb Haemost* 2007;**97**:822–9.

62. Tisoncik JR, Korth MJ, Simmons CP *et al.* Into the Eye of the Cytokine Storm. *Microbiol Mol Biol Rev MMBR* 2012;**76**:16–32.

63. Banerji A, Riedl M. Managing the Female Patient with Hereditary Angioedema. *Womens Health* 2016;**12**:351–61.

64. Lunn M. Does The Severity And Frequency Of Hereditary Angioedema Attacks Change With Age? A Patient Perspective. *J Allergy Clin Immunol* 2010;**125**:AB168.

65. Cesari M, Pahor M, Incalzi RA. Plasminogen activator inhibitor-1: A Key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc Ther* 2010;**28**:e72–91.

66. Ferrucci L, Corsi A, Lauretani F *et al.* The origins of age-related proinflammatory state. *Blood* 2005;**105**:2294–9.

67. EMA. Takhzyro. *Eur Med Agency* 2018.

68. Research C for DE and. FDA approves new treatment for rare hereditary disease. *FDA* 2021.

69. Fijen LM, Riedl MA, Bordone L *et al.* Inhibition of Prekallikrein for Hereditary Angioedema. *N Engl J Med* 2022;**386**:1026–33.

70. Porebski G, Kwitniewski M, Reshef A. Biomarkers in Hereditary Angioedema. *Clin Rev Allergy Immunol* 2021;**60**:404–15.

71. Zuraw BL, Maurer M, Sexton DJ *et al.* Therapeutic monoclonal antibodies with a focus on hereditary angioedema. *Allergol Int* 2022, DOI: 10.1016/j.alit.2022.06.001.

72. Cohn DM, Viney NJ, Fijen LM *et al.* Antisense Inhibition of Prekallikrein to Control Hereditary Angioedema. *N Engl J Med* 2020;**383**:1242–7.

73. Perneger TV. What's wrong with Bonferroni adjustments. *BMJ* 1998;**316**:1236–8.

Figure legends:

Figure 1. Pathways

An overview of the systems affected by C1 inhibitor deficiency in which the contact and kallikrein-kinin systems are dysregulated. Downstream, this leads to increased activation of procoagulant and proinflammatory mediators. There are also significant interactions between these mediators, resulting in increased thromboinflammation.

TAT = Thrombin-antithrombin complexes. F1+2 = Prothrombin fragment 1+2. FGF2 = fibroblast growth factor 2. β-TG = β-thromboglobulin. CCL = C-C chemokine ligand. PDGF-BB = platelet derived growth factor BB. PAI-1 = Plasminogen activator inhibitor-1. TNF = Tumour necrosis factor. IL = interleukin. CXCL = C-X-C chemokine ligand. IL = interleukin. G-CSF = granulocyte colony stimulating factor. GM-CSF = granulocyte-macrophage colony stimulating factor.

Figure 2. Complement:

Plasma levels of C4d, C3bc and the soluble terminal C5b-9 complex (TCC) are shown. Scatter plot: Each dot represents a patient or control. The middle horizontal line represents the median, and the upper and lower horizontal bars represent the upper and lower limits of the interquartile range, respectively. N = 20 in each group. AU = arbitrary units.

Uncorrected p-values are displayed above, Holm-Bonferroni corrected p-values are displayed in parentheses below.

Figure 3. Haemostasis:

Plasma levels of β-TG, F1+2, TAT and PAI-1 are shown.

β-TG = β-thromboglobulin. F1+2 = Prothrombin fragment 1+2. TAT = Thrombin-antithrombin complexes. PAI-1 = Plasminogen activator inhibitor-1.

Scatter plot: Each dot represents a patient or control. The middle horizontal line represents the median, and the upper and lower horizontal bars represent the upper and lower limits of the interquartile range, respectively. N = 20 in each group. Uncorrected p-values are displayed above, Holm-Bonferroni corrected p-values are displayed in parentheses below.

Figure 4. Neutrophils:

Plasma levels of myeloperoxidase (MPO) are shown.

Scatter plot: Each dot represents a patient or control. The middle horizontal line represents the median, and the upper and lower horizontal bars represent the upper and lower limits of the interquartile range, respectively. N = 20 in each group. Uncorrected p-values are displayed above, Holm-Bonferroni corrected p-values are displayed in parentheses below.

Figure 5. Interleukins:

Plasma levels of 12 interleukins are shown.

TNF = Tumour necrosis factor. IL = interleukin.

Scatter plot: Each dot represents one patient or control. The middle horizontal line represents the median, and the upper and lower horizontal bars represent the upper and lower limits of the interquartile range, respectively. N = 20 in each group. Uncorrected p-values are displayed above, Holm-Bonferroni corrected p-values are displayed in parentheses below.

Figure 6. Chemokines:

Plasma levels of CXCL-8, eotaxin, CXCL10, CCL2, CCL3 and CCL4 are shown. CXCL = C-X-C chemokine ligand. IL = interleukin. CCL = C-C chemokine ligand. IP = interferon-γ inducible protein. MCP = Monocyte Chemoattractant Protein. MIP = macrophage inflammatory protein.

Scatter plot: Each dot represents a patient or control. The middle horizontal line represents the median, and the upper and lower horizontal bars represent the upper and lower limits of the interquartile range, respectively. N = 20 in each group. Uncorrected p-values are displayed above, Holm-Bonferroni corrected p-values are displayed in parentheses below.

Figure 7. Regulators, growth factor and interferons:

Plasma levels of IL-1RA, INF-γ, FGF2, G-CSF, GM-CSF and PDGF-BB are shown. IL-1RA = IL-1 receptor antagonist. G-CSF = granulocyte colony stimulating factor. GM-CSF = granulocyte-macrophage colony stimulating factor. FGF2 = fibroblast growth factor 2. PDGF-BB = platelet derived growth factor BB. IFN = interferon.

Scatter plot: Each dot represents a patient or control. The middle horizontal line represents the median, and the upper and lower horizontal bars represent the upper and lower limits of the interquartile range, respectively. N = 20 in each group. Uncorrected p-values are displayed above, Holm-Bonferroni corrected p-values are displayed in parentheses below.

Figure 8. Subgroup analyses:

Plasma levels of MPO, IL-2, IL-4, IL-5, IL-13, Eotaxin, CCL2, CCL4 and GM-CSF are shown.

IL = interleukin. CCL = C-C chemokine ligand. GM-CSF = granulocyte-macrophage colony stimulating factor.

Scatter plot: Each dot represents a patient in its respective subgroup. The middle horizontal line represents the median, and the upper and lower horizontal bars represent the upper and lower limits of the interquartile range, respectively.

N = 11 for HAE female, 9 for HAE male; 13 for HAE type 1, 7 for HAE type 2; 10 for patients defined as being on prophylaxis and 10 for patients defined as not being on prophylaxis.

Uncorrected p-values are displayed above, Holm-Bonferroni corrected p-values are displayed in parentheses below.

Figure 1

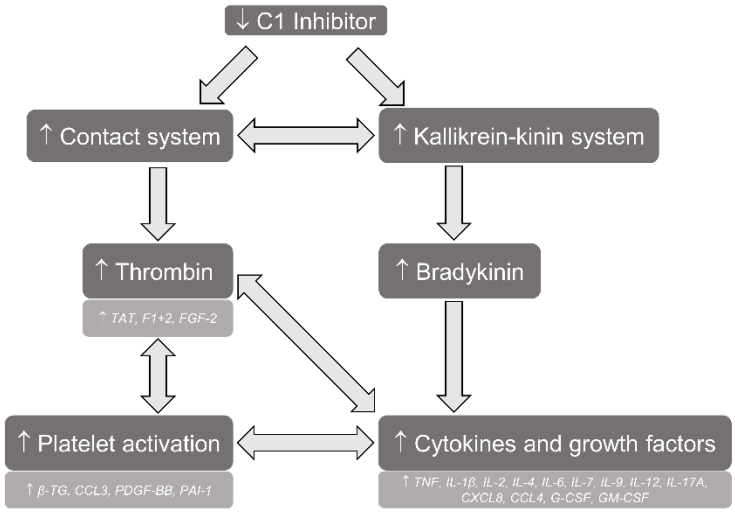
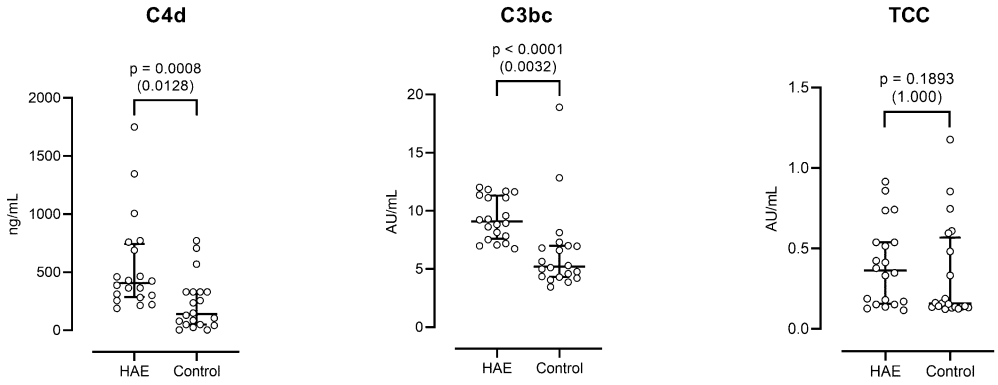


Figure 2



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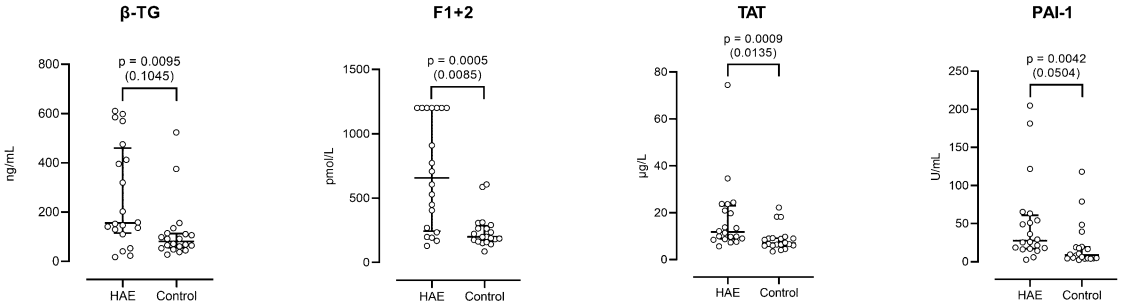

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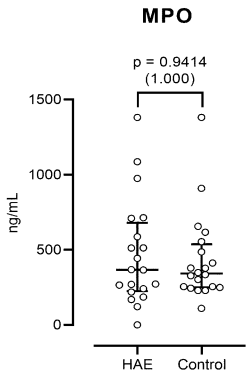

Figure 5

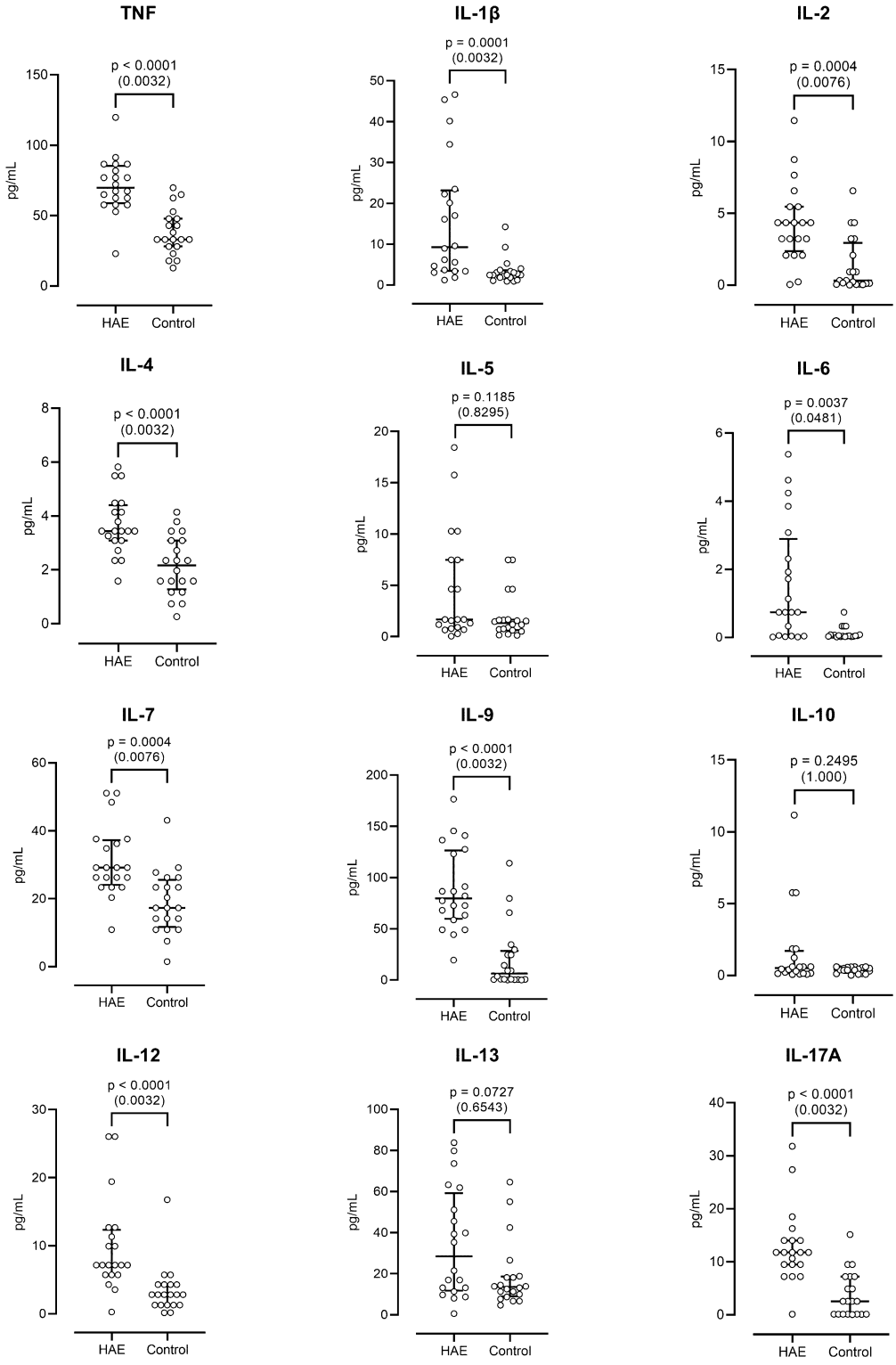

Figure 6

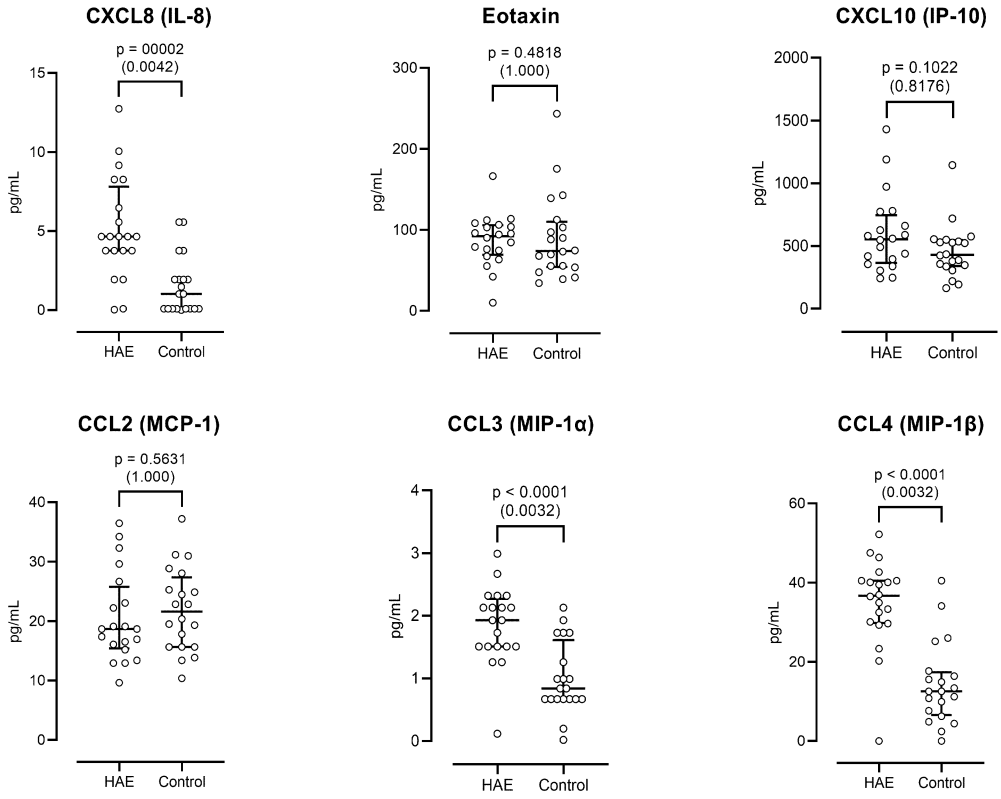

Figure 7

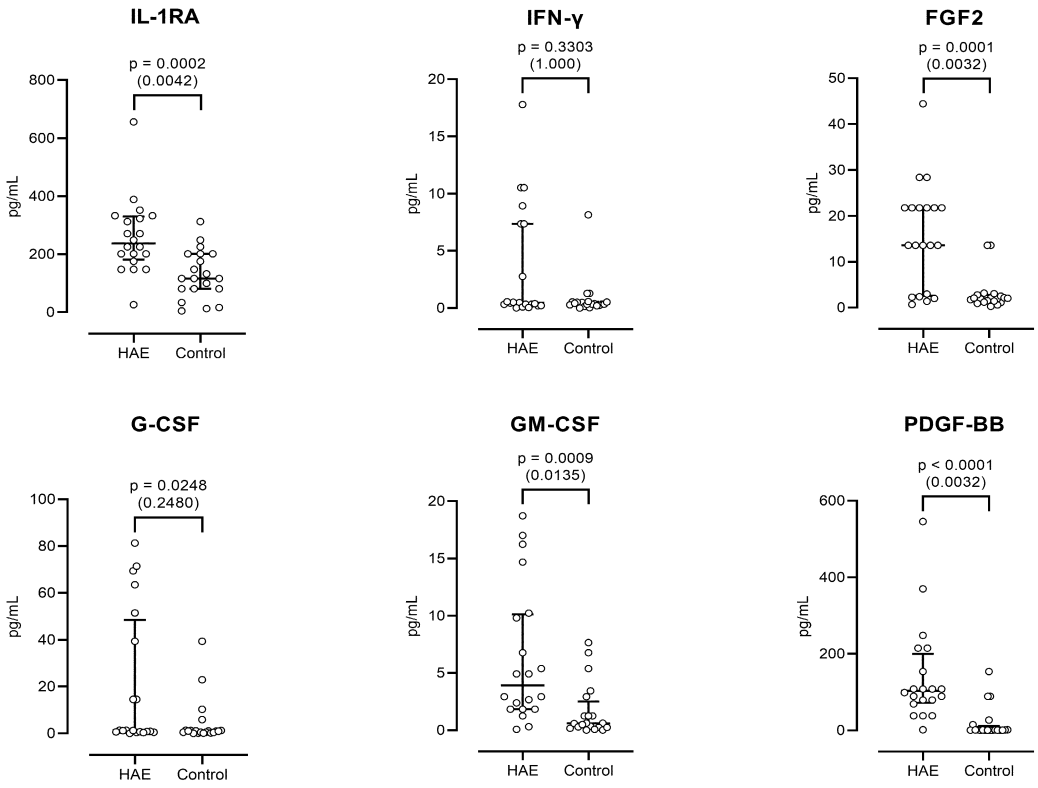

Figure 8