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Altered levels of phospholipases C, diacylglycerols, endocannabinoids and N -acylethanolamines in patients with hereditary angioedema due to FXII mutation

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Abstract:	**Background:** Hereditary angioedema (HAE) is a rare genetic disorder characterized by local, self-limiting edema due to temporary increase in vascular permeability. HAE with normal C1 esterase inhibitor (C1INH) activity includes the form with mutations in the *F12* gene encoding for coagulation factor XII (FXII-HAE) causing an overproduction of bradykinin (BK) leading to angioedema attack. BK binding to B2 receptors (BK2R) leads to an activation of phospholipase C (PLC) and subsequent generation of second messengers: diacylglycerols (DAGs) and possibly the endocannabinoids (eCBs) 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and eCB-related *N*-acylethanolamines [palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)]. To date, there are no data on the role of these lipid mediators in FXII-HAE. **Methods:** Here, we analyzed plasma levels of PLC, DAG and eCBs in 40 patients with FXII-HAE and 40 sex and age-matched healthy individuals. **Results:** Plasma PLC activity was increased in FXII-HAE patients compared to controls. Concentrations of DAG 18:1-20:4, a lipid second messenger produced by PLC, were higher in FXII-HAE compared to controls, and positively correlated with PLC activity and cleaved high molecular kininogen (cHK). Also the concentrations of the DAG metabolite, 2-AG were altered in FXII-HAE. AEA and OEA were decreased in FXII-HAE patients compared to controls; by contrast, PEA, was increased. The levels of all tested mediators did not differ between symptomatic and asymptomatic patients. Moreover, C1INH-HAE patients had elevated plasma levels of PLC, that correlated with cHK, but the levels of DAGs and eCBs were the same as controls. **Conclusions:** BK overproduction and BKR2 activation are linked to alteration of PLCs and their metabolites in patients with FXII-HAE. Our results may pave the way to investigations on the functions of these mediators in the pathophysiology of FXII-HAE, and provide new potential biomarkers and therapeutic targets.

Altered levels of phospholipases C, diacylglycerols, endocannabinoids and *N*-acylethanolamines in patients with hereditary angioedema due to FXII mutation

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Short Title: Lipid mediators in FXII-HAE

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ABSTRACT

Background: Hereditary angioedema (HAE) is a rare genetic disorder characterized by local, self-limiting edema due to temporary increase in vascular permeability. HAE with normal C1 esterase inhibitor (C1INH) activity includes the form with mutations in the *F12* gene encoding for coagulation factor XII (FXII-HAE) causing an overproduction of bradykinin (BK) leading to angioedema attack. BK binding to B2 receptors (BK2R) leads to an activation of phospholipase C (PLC) and subsequent generation of second messengers: diacylglycerols (DAGs) and possibly the endocannabinoids (eCBs) 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and eCB-related *N*-acylethanolamines [palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)]. To date, there are no data on the role of these lipid mediators in FXII-HAE.

Methods: Here, we analyzed plasma levels of PLC, DAG and eCBs in 40 patients with FXII-HAE and 40 sex and age-matched healthy individuals.

Results: Plasma PLC activity was increased in FXII-HAE patients compared to controls. Concentrations of DAG 18:1-20:4, a lipid second messenger produced by PLC, were higher in FXII-HAE compared to controls, and positively correlated with PLC activity and cleaved high molecular kininogen (cHK). Also the concentrations of the DAG metabolite, 2-AG were altered in FXII-HAE. AEA and OEA were decreased in FXII-HAE patients compared to controls; by contrast, PEA, was increased. The levels of all tested mediators did not differ between symptomatic and asymptomatic patients. Moreover, C1INH-HAE patients had elevated plasma levels of PLC, that correlated with cHK, but the levels of DAGs and eCBs were the same as controls.

Conclusions: BK overproduction and BKR2 activation are linked to alteration of PLCs and their metabolites in patients with FXII-HAE. Our results may pave the way to investigations on the functions of these mediators in the pathophysiology of FXII-HAE, and provide new potential biomarkers and therapeutic targets.

KEY WORDS

2-arachidonoylglycerol, anandamide, diacylglycerols, oleoylethanolamide, palmitoylethanolamide.

ABBREVIATIONS

2-arachidonoylglycerol, 2-AG; anandamide, AEA; arachidonic acid, AA; bradykinin receptor, BKR2; bradykinin, BK; C1 esterase inhibitor, C1INH; cleaved high molecular weight kininogen, cHK; DAG lipases, DAGLs; diacylglycerols, DAGs; endocannabinoids, eCBs; fluoro-enzyme immune assay, FEIA; FXII, factor XII; hereditary angioedema, HAE; *N*-acyl-ethanolamines, NAEs; oleoylethanolamide, OEA; palmitoylethanolamide, PEA; phospholipase enzymes C, PLCs; protease inhibitor cocktail, PIC; protein kinase C, PKC; secreted phospholipase A_2, PLA_2; sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE

INTRODUCTION

Hereditary angioedema (HAE) is a rare autosomal dominant genetic disorder characterized by local, self-limiting edema due to temporary and periodic increase in vascular permeability. Affected individuals suffer from chronically recurrent swelling episodes localized to the skin and/or to the mucous membranes of the upper respiratory and gastrointestinal tracts (1-3). Inappropriate control of the contact system, consisting of factor XII (FXII), plasma kallikrein and high molecular weight kininogen (HK), results in overproduction of bradykinin (BK), which increases vascular permeability and thus induces angioedema attacks. The most common form of HAE is caused by deficiency of C1 esterase inhibitor (C1INH-HAE), but HAE can also occur with normal plasma levels of C1INH (nlC1INH-HAE). Specifically, among nlC1INH-HAE we can recognize: plasminogen (PLC-HAE), angiopoietin 1 (ANGPT1-HAE), kininogen (KNG-HAE), myoferlin (MYOF-HAE), heparan-sulfate-glucosamine-3-O-sulfotrasferase 6 (HS3ST6-HAE) (3). In addition, one of these forms of HAE can be due to mutations in the gene coding for coagulation FXII (*F12*, FXII-HAE) (4).

To date, four pathogenic variants in the *F12* gene have been identified, all located in the highly glycosylated proline-rich region (PRR) adjacent to the Arg372-Val373 bond that is cleaved by plasma kallikrein during FXII activation (5). The FXII-HAE phenotype is almost expressed by females compared to males due to the fluctuation in the estrogen levels during pregnancy or oral contraceptive use (6). The increased levels of estrogens favor a huge production of FXII due to the presence of an estrogen-response element in the promoter region of the *F12* gene leading to FXII activation and BK production (7).

F12 mutations introduce a cleavage site for plasmin that facilitates conversion of FXII protein into its active form FXIIa, which can in turn generate active kallikrein and BK leading to angioedema attack (8). The broad spectrum of BK (patho)physiological functions is mediated by G protein-coupled receptors (GPCRs), known as B1 and B2 subtypes. BK preferentially binds to B2 receptors (BK2R) constitutively expressed in many tissues (9). In cells analyzed until now, the activation of BK receptors, mediated by a G protein pathway, leads to an activation of phospholipase C (PLC) and

subsequent generation of second messengers, such as diacylglycerols (DAGs) (9-11). DAGs are physiological activators of protein kinase C (PKC) and in the case of *sn*-2-arachidonoyl-DAG species, they are also precursors of the endocannabinoid 2-arachidonoylglycerol (2-AG) through the action of DAG lipases (DAGLs) (11). 2-AG acts preferentially on cannabinoid receptors but can also be an alternative precursor of arachidonic acid (AA) and its vasoactive mediators, the eicosanoids (11, 12). The eCBs 2-AG and anandamide (AEA), together with the eCB-related AEA congeners, i.e. *N*-acylethanolamines (NAEs) like oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), are biosynthesized "on demand" from membrane phospholipids, although NAEs are normally produced via PLC-independent pathways (13). For all those mediators, it has been described a role on vasculature. AEA is reported to play an important role as vasorelaxant in several context (27633407, 22627170, 10788462), together with OEA (23340219). In addition, AEA and OEA are able to decrease blood–brain barrier permeability (25651941). 2-AG impairs endothelial repair and promotes leukocyte–endothelial cell adhesion (32810540).

Owing to overproduction of BK in FXII-HAE and the ability of BK to activate *via* BK2R the pathway of PLCs, DAGs and 2-AG, and the relationship of the latter with NAEs, we have analyzed the plasma concentration or activity of these lipid mediators in patients with FXII-HAE.

METHODS

Study population

We studied 40 adult Caucasian patients with FXII-HAE (5 males and 35 females; age range: 4–92 years; median age 39 years) 29 of which were symptomatic, followed at the University of Naples Federico II (Italy), University of Cagliari (Italy), Hospital La Paz Institute for Health Research of Madrid (Spain) and at Johannes Gutenberg University of Mainz (Germany). All of them carried the p.Thr328Lys mutation of the FXII. Forty healthy individuals (5 males and 35 females; age range: 8-85 years; median age 39 years), all Caucasians, were studied as control group enrolled at the University of Naples Federico II. Inclusion criteria of FXII-HAE patients were: normal values of

activity of C1INH (>50%), normal concentration C1INH and C4 and mutation in the *F12* gene. All patients enrolled in this study reported absence of any known chronic or acute pathological condition at the time of enrollment and expression of written informed consent for genetic and clinical studies. Exclusion criteria of FXII-HAE patients and control group were: presence of any condition that, in the opinion of the investigator, could interfere with the completion of the study procedures (e.g. pregnancy). In a next step of the study we enrolled 26 C1INH-HAE patients at the University of Naples Federico II (Italy) (5 males and 21 females; age range: 27-70 years; median age 42.6 years) and we have sex- and age-matched them with FXII-HAE patients and healthy controls previously enrolled. Inclusion criteria of C1INH-HAE patients were: recurrent angioedema attacks, low values of activity of C1INH (<50%), low concentration of protein C1INH and C4 and/or mutation in the *SERPING1* gene, which encodes for C1INH.

Plasma collection and Ethical Aspects

The Ethical Committee (protocol number PT 1553/18) approved that plasma obtained during routine diagnostics could be used for research investigating the pathophysiology of angioedema and written informed consent was obtained from patients according to the principles expressed in the Declaration of Helsinki. The controls had been referred for routine medical check-up and volunteered for the study by giving informed consent.

Complement parameters

Blood samples were diluted with sodium citrate solution (0.11 mol/L) and then centrifuged (20 min, 2000 ×g, 22 °C). The plasma samples collected were immediately frozen and stored at -80 °C until tested. C1INH activity was measured using a colorimetric assay (Technochrome C1INH, Technoclone GmbH, Vienna, Austria). Normal values of activity of C1INH are greater than 0.7 Unit

C1INH/mL (>70%). C1INH and C4 antigen levels were measured by means of radial immunodiffusion (RID) (NOR-Partigen, Siemens Healthcare Diagnostics, Munich, Germany).

Cleavage of High-Molecular Weight Kininogen

Measurements were conducted collecting blood in tubes containing sodium citrate, tubes containing the protease inhibitors cocktail (PIC) previously described (14) and commercial tubes (BD Sodium citrate, code 363080) with PIC added by the manufacturer. PIC prevents *in vitro* activation of contact system that occurs during blood collection and handling. Blood samples from all patients were obtained at least 8 days apart from an angioedema attack. The cleavage of HK was assessed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis (a modification of the method described by Berrettini *et al*.) (15, 16). The amount of cleaved HK (cHK) was expressed as a percentage of total HK (17).

Phospholipases C activity assay

PLC activity was determined using EnzChek® Direct Phospholipase C Assay kit (Life-technologies). Results are expressed as units/L of PLC activity.

Measurement of eCBs (AEA, 2-AG), NAEs (PEA, OEA) and DAGs

Plasma was sonicated and extracted with chloroform/methanol/Tris–HCl 50 mmol/L pH 7.5 (2:1:1, vol/vol) containing internal standards ([H_2]8 AEA 5 pmoL; [H_2]5 2-AG, [H_2]5 PEA and [H_2]4 OEA 50 pmoL each) for eCBs and NAE quantification as well as 1,2-heptadecanoin (Larodan AB, Malmo, Sweden) for DAG measurement. The lipid containing organic phase was dried down, weighed, and pre-purified by open-bed chromatography on silica gel with 99:1, 90:10 and 50:50 (*v/v*) chloroform/methanol. The 90:10 fraction was used for eCBs and NAE quantification by LC-APCI-

MS (LCMS-2020 Shimadzu) as previously reported (18). DAG levels were measured by LC-MS-MS using an LC20AB coupled to a hybrid detector IT-TOF (Shimadzu Corporation, Kyoto, Japan) equipped with an ESI interface (19).

Statistical analysis

Data were analysed with the GraphPad Prism 5 software package. Data were tested for normality using the D'Agostino-Pearson normality test. If normality was not rejected at 0.05 significance level, we used parametric tests. Otherwise, for not-normally distributed data we used nonparametric tests. Statistical analysis was performed by unpaired two-tailed t-test or two-tailed Mann-Whitney test as indicated in figure legends. Correlations between two variables were assessed by Spearman's correlation analysis and reported as coefficient of correlation (r). A p value ≤ 0.05 was considered statistically significant. Plasma levels of PLA_2, PLC, DAGs and eCBs are shown as the mean (horizontal black line) of controls, FXII-HAE and C1INH-HAE patients.

RESULTS

PLC plasma activity and DAG 18:1-20:4 concentrations are increased in patients with FXII-HAE

We measured plasma PLC activity in patients with FXII-HAE (N=40) and age and sex matched healthy controls (N=40) (Fig.1). PLC (Fig. 1A) activity was increased in patients with FXII-HAE compared to controls [healthy controls: PLC = 0.09±0.01 U/mL ± ES; FXII-HAE: PLC = 0.26±0.01 U/mL ± ES].

To evaluate whether the enhancement of PLC activity was accompanied by an increased production of DAGs, we measured DAG 18:1-20:4 and DAG 18:0-20:4 concentrations in the plasma of FXII-HAE patients. Figure 1 shows that DAG 18:1-20:4 (panel B), but not DAG 18:0-20:4 (panel C), concentrations in the plasma of FXII-HAE patients was higher than in healthy controls [healthy controls: DAG 18:1-20:4 = 65.05±7.13 pmol/mL ± ES; FXII-HAE: DAG 18:1-20:4 = 176.1±48.81 pmol/mL ± ES] [healthy controls: DAG 18:0-20:4 = 653.3±122.8 pmol/mL ± ES; FXII-HAE: DAG 18:0-20:4 = 767.3±309.5 pmol/mL ± ES]. Similar increase was noted when expressing DAG 18:1-20:4 and 18:0-20:4 concentrations as pmol/mg of lipid extract (see supplementary Figure 1) [healthy controls: DAG 18:1-20:4 = 11.3±1.2 pmol/mg of lipid extracts ± ES; FXII-HAE: DAG 18:1-20:4 = 26.5±6.9 pmol/mg of lipids extracts ± ES] [healthy controls: DAG 18:0-20:4 = 93.5±13.8 pmol/ mg of lipid extracts ± ES; FXII-HAE: DAG 18:0-20:4 = 98.6±36.7 pmol/ mg of lipid extracts ± ES].

There was no correlation between the age and PLC activity or DAG 18:1-20:4 concentrations in either patients or controls (data not shown).

DAG 18:1-20:4 and DAG 18:0-20:4 concentrations were positively correlated with each other in both healthy controls (Fig. 2A) and FXII-HAE patients (Fig. 2B). Moreover, unlike healthy controls (Fig. 2C), PLC activity was positively correlated with DAG 18:1-20:4 concentrations in FXII-HAE patients (Fig. 2D).

Endocannabinoids and related mediators in patients with FXII-HAE

Similar to the concentration of one of its major biosynthetic precursors (DAG 18:1-20:4) (see above), 2-AG concentrations were higher in patients with FXII-HAE compared to controls (Fig. 3A) [healthy controls: 2-AG = 15.6±3.8 pmol/mL ± ES; FXII-HAE: 2-AG = 21.2±4.0 pmol/mL ± ES], although they did not correlate positively with DAG concentrations (Fig. 3B) and PLC activity (Fig. 3C). Interestingly, AEA concentrations were lower in patients with FXII-HAE compared to controls (Fig. 3D) [healthy controls: AEA = 7.1±1.2 pmol/mL ± ES; FXII-HAE: AEA = 3.8±0.3 pmol/mL ± ES]. By contrast PEA concentrations were increased in FXII-HAE (Fig. 3E) [healthy controls: PEA = 19.7±1.5 pmol/mL ± ES; FXII-HAE: PEA = 26.9±1.7 pmol/mL ± ES]. OEA concentrations, similar to AEA, were decreased in FXII-HAE (Fig. 3F) [healthy controls: OEA = 13.8±1.2 pmol/mL ± ES; FXII-HAE: OEA = 9.4±1.0 pmol/mL ± ES].

No correlation was found between age and eCB and NAE concentrations in either patients or healthy controls (data not shown).

Relationships among PLC, DAGs and eCBs and disease characteristics

To understand whether altered concentrations of PLC and their metabolites reflected different clinical and laboratory characteristics, we used two experimental analyses. First, we analyzed the correlation among lipid metabolites and cHK because patients with FXII-HAE exhibit markedly elevated plasma levels of cHK compared to healthy controls (1). cHK is an indirect measure of the bradykinin released upon activation of the contact system. We measured plasma levels of cHK in samples from 13 healthy subjects and 13 patients with FXII-HAE. Fig 4A confirms that FXII-HAE had elevated amounts of cHK compared to controls [healthy controls: cHK = 25±1.05 cHK (% of total HK) ± ES; FXII-HAE: cHK = 51.8±1.97 cHK (% of total HK) ± ES]. PLC (Fig. 4B) and DAG 18:1-20:4 (Fig. 4C) positively correlated with cHK in FXII-HAE patients. By contrast, cHK did not correlate with 2-AG, AEA, PEA, OEA levels (supplementary Fig. 2A-D).

Finally, we grouped FXII-HAE patients according to their clinical symptoms into two groups: asymptomatic (N=11) and symptomatic patients (N=29). The concentrations of PLC, DAGs, 2-AG, AEA, PEA and OEA were compared among the groups. Supplementary figure 3 shows that there were no differences in the levels of PLC (A), DAGs (B, C), 2-AG (D), PEA (F)and OEA (G)between asymptomatic and symptomatic FXII-HAE patients. Only AEA concentrations were higher in asymptomatic than symptomatic patients (Suppl. Fig. 3E) [asymptomatic FXII-HAE: AEA = 5.12±0.94 pmol/mL ± ES; symptomatic FXII-HAE: AEA= 3.3±0.38 pmol/mL ± ES].

PLC, DAGs and eCBs in patients with C1INH-HAE

In a last step of this project we enrolled 26 patients with C1INH-HAE and measured their plasma PLC, DAGs, eCBs and NAEs and compared them with age and sex matched healthy controls (N=26) and FXII-HAE patients (N=26). PLC activity, similar to FXII-HAE, is increased in patients with C1INH-HAE compared to controls [healthy controls: PLC = 0.01±0.01 U/mL ± ES; C1INH-HAE: PLC= 0.2±0.01 U/mL ± ES; FXII-HAE: PLC = 0.25±0.02 U/mL ± ES] (Fig. 5A) and positively correlated with cHK (Fig. 5B). By contrast, the concentrations of DAG 18:1-20:4 (Fig. 5C), DAG 18:0-20:4 (Fig. 5D), 2-AG (Fig. 5E), AEA (Fig. 5F), PEA (Fig. 5G) and OEA (Fig. 5H) were not increased in C1INH-HAE patients compared to controls.

DISCUSSION

In this study, we describe for the first time that plasma PLC activity and concentrations of their metabolites (e.g. DAG 18:1-20:4 and 2-AG) are significantly altered in patients with FXII-HAE. These patients exhibit: 1) increased plasma activity of PLC; 2) elevated DAG 18:1-20:4, 2-AG, and PEA concentrations, and 3) decreased levels of AEA and OEA.

It is well known that BK activates BKR2 inducing PLC activity and lipid mediators production, and this observation is in line with the results of our study, showing that some of these molecules, in particular PLC, are positively correlated with cHK, which is elevated in FXII-HAE patients.

BK, generated by proteolytic cleavage of its precursor HK, is considered to be the main mediator of HAE (2). It is well explained how the kallikrein-kinin system leads to BK production: the binding of negatively charged molecules to FXII induces a conformational change causing its activation. Activated FXII converts prekallikrein to kallikrein, which cleaves HK to release BK (20). FXII-HAE is caused by a mutation of *F12* gene that generates a mutant FXII protein more sensitive to enzymatic cleavage by plasmin (21, 22). These FXII mutants are rapidly cleaved by plasmin at the site of mutation leading to the formation of a smaller factor XII termed "δ factor XII". The δ factor is converted to an active enzyme by plasma kallikrein (or plasmin) so rapidly, it escapes inhibition by C1INH eliciting excessive BK formation. In fact, we have described previously that levels of cHK, an indirect measure of the BK released upon activation of the contact system, are higher in plasma of FXII-HAE patients compared to normal subjects (1). We also reconfirmed these data in the study population of this paper. The binding between BK and BKR2, expressed on several cells including endothelial cells (9, 20), activates an intracellular signal cascade leading to activation of PLC and subsequent generation of second messengers, such as DAGs (9-11). Here, we demonstrate that PLC activity is elevated in FXII-HAE patients compared to controls and is positively correlated with cHK plasma levels. We have obtained similar results in C1INH-HAE patients. Additionally, the levels of cHK were higher in highly symptomatic C1INH-HAE patients than in those with less frequent attacks

(16), whereas, by contrast, in FXII-HAE patients both cHK concentrations and PLC activity did not differ between symptomatic and asymptomatic patients, suggesting that PLC may not be involved in the development of FXII-HAE attack. In line with the literature (1, 5), the majority of our FXII-HAE patients are women (87.5%) and among the symptomatic cases (72.5%), the 96.5% are female. Indeed, men carrying the mutation are more often asymptomatic than women (1, 5), and in fact in our study population 80% men *vs* 20% women are asymptomatic.

PLC products such as DAG 18:1-20:4, but not DAG 18:0-20:4, were increased in patients with FXII-HAE and positively correlated with PLC activity, although their concentrations are similar in symptomatic and asymptomatic patients. Interestingly, DAG 18:1-20:4 concentrations are not correlated with those of its metabolite 2-AG, even though 2-AG concentrations are increased in FXII-HAE patients. Other sources of DAGs and/or DAG-independent biosynthetic pathways for 2-AG (possibly due to shortage of DAG lipase activity), might explain this finding. It is conceivable that the increased DAG concentrations in FXII-HAE could reflect also an altered PKC activation, which is a promiscuous second messenger cascade regulating vasodilator responses in endothelial cells (23). For instance, PKC may mediate endothelial nitric oxide (NO) synthesis and promote vasodilation (24). Despite, the fact that NO levels are increased in C1INH-HAE patients (25), we have not found altered levels of DAGs and 2-AG in these patients. In the future, it would be interesting to investigate PKC and NO concentrations in FXII-HAE to understand if DAG level elevation affects PKC and NO production. Regardless of the mechanism(s) underlying 2-AG production, this phenomenon, and the subsequent activation of CB1 or CB2 receptors, might represent an adaptive response to FXII-HAE, by virtue of the analgesic and anti-inflammatory actions, respectively, of these two receptors (26). Similarly to eCB 2-AG, also the AEA and its NAE congeners PEA and OEA (27) were altered in FXII-HAE but not in C1INH-HAE patients. These molecules are involved in endogenous, cannabinoid receptor-dependent and independent, protective mechanisms that are activated as a result of different types of tissue damage or stimulation of inflammatory responses (28).

Strong evidence indicates that PEA has anti-inflammatory actions (12, 29); thus, the increase of PEA plasma levels in FXII-HAE could represent an attempt to activate the Th2-mediated anti-inflammatory immune response. By contrast the decrease of AEA and OEA concentrations, , which are endowed of analgesic, anti-inflammatory and vasodilatory effects mediated by CB1, CB2, PPARα or other receptors, could contribute to the symptoms of this disorder. Interestingly, previous studies (30) have emphasized how the levels of 2-AG and AEA are often regulated in opposing manners, possibly due to the different enzymes involved in the biosynthesis and inactivation of these two eCBs.

PLC and the metabolites measured in this study are produced from several immune cells (31, 32). Our data show that both PLC, DAGs and eCBs and related NAEs are altered in plasma of patients with FXII-HAE. On the other hand, their cellular sources remain unknown and further studies are needed to understand the origin of such mediators in these patients.

Unquestionably, the overproduction of BK due to known pathogenic mutation in p.T328K *F12* gene mutation induces a massive intracellular signaling cascade that causes an abnormal PLC activation and PLC metabolites production. Further studies are needed to evaluate the diagnostic and prognostic value of PLs, DAGs, eCBs and NAEs in FXII-HAE, and to understand whether pharmacological blockade of these mediators (e.g. PKC, CB1, CB2, PPARα, etc.) improves or exacerbates the symptoms of angioedema.

STATEMENT OF CONFLICTS OF INTEREST

None

LIST OF AUTHOR CONTRIBUTIONS

Participate in research design: Loffredo S., Ferrara A.L., Piscitelli F., Palestra F., Petraroli A., Suffritti C., Firinu D., López-Lera A., Caballero T., Bork K., Spadaro G., Di Marzo V., Bova M.

Conducted experiments: Loffredo S., Ferrara A.L., Piscitelli F., Palestra F., Suffritti C., López-Lera A.

Clinical enrollment: Petraroli A., Firinu D., Caballero T., Bork K., Spadaro G., Bova M.

Performed data analysis: Loffredo S., Ferrara A.L., Piscitelli F., Palestra F., Suffritti C.

Wrote or contributed to the writing the manuscript: Loffredo S., Ferrara A.L., Piscitelli F., Palestra F., Petraroli A., Suffritti C., Firinu D., López-Lera A., Caballero T., Bork K., Spadaro G., Di Marzo V., Bova M.

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DATA AVALIABILITY

All data are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Figure 1. Activity of PLC, and DAG 18:1-20:4 and 18:0-20:4 concentrations in plasma of patients with FXII-HAE and healthy controls.

Plasma activity of PLC (A), DAG 18:1-20:4 (B) and 18:0-20:4 (C) concentration expressed in pmol/mL in 40 controls (Healthy) and in 40 patients with FXII-HAE in remission was determined by EIA and LC-MS-MS. Horizontal bars depict the median value. A p-value ≤ 0.05 was considered statistically significant.

Figure 2. Correlation among DAG 18:1-20:4, DAG 18:0-20:4 levels and PLC activity in plasma of patients with FXII-HAE and healthy controls.

Correlation between two variables: DAG 18:1-20:4 and DAG 18:0-20:4 in Healthy (A) and FXII-HAE (B) and DAG 18:1-20:4 and PLC in Healthy (C) and FXII-HAE (D) were assessed by Spearman's correlation analysis and reported as coefficient of correlation (r). *p*-value ≤ 0.05 was considered statistically significant.

Figure 3. 2-AG, AEA, PEA and OEA concentrations in plasma of patients with FXII-HAE and healthy controls.

(A) 2-AG concentration was determined in 40 controls (Healthy) and in 40 patients with FXII-HAE in remission. Horizontal bars depict the median value. Correlations between 2-AG and DAG 18:1-20:4 in FXII-HAE (B) and 2-AG and PLC activity in FXII-HAE (C) were assessed by Spearman's correlation analysis and reported as coefficient of correlation (r). *p*-value ≤ 0.05 was considered statistically significant.

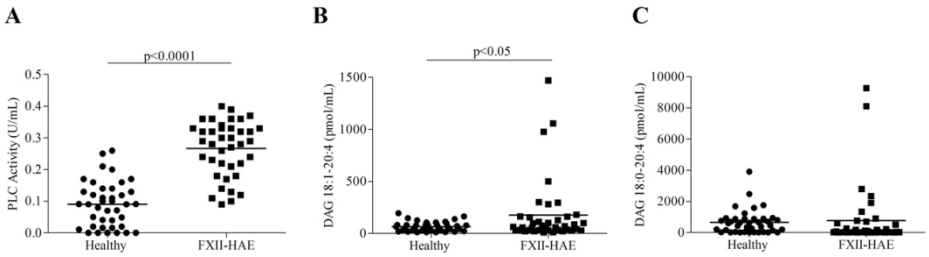
AEA (D), PEA (E) and OEA (F) concentrations in 40 controls (Healthy) and in 40 patients with FXII-HAE in remission. Horizontal bars depict the median value. *p*-value ≤ 0.05 was considered statistically significant.

Figure 4. cHK levels and correlation among PLC, DAG 18:1-20:4 and cHK in plasma of patients with FXII-HAE.

Percentage of cHK (A) and correlation between two variables: PLC and cHK (B), DAG 18:1-20:4 and cHK (C) in 13 FXII-HAE patients were assessed by Spearman's correlation analysis and reported as coefficient of correlation (r). p-value ≤ 0.05 was considered statistically significant.

Figure 5. Levels of PLC, DAGs, eCBs and NAEs in plasma of patients with C1INH-HAE

Plasma activity of PLC (A), correlation between PLC and cHK (B), DAG 18:1-20:4 (C), DAG 18:0-20:4 (D), 2-AG (E), AEA (F), PEA (G), OEA (H) concentrations, was determined in 26 controls (Healthy),in 26 patients with C1INH-HAE and 26 patients with FXII-HAE in remission. Horizontal bars depict the median value (A, C-H). Correlation was measured by Spearman's correlation analysis and reported as coefficient of correlation (*r*). (B). A *p*-value ≤ 0.05 was considered statistically significant.



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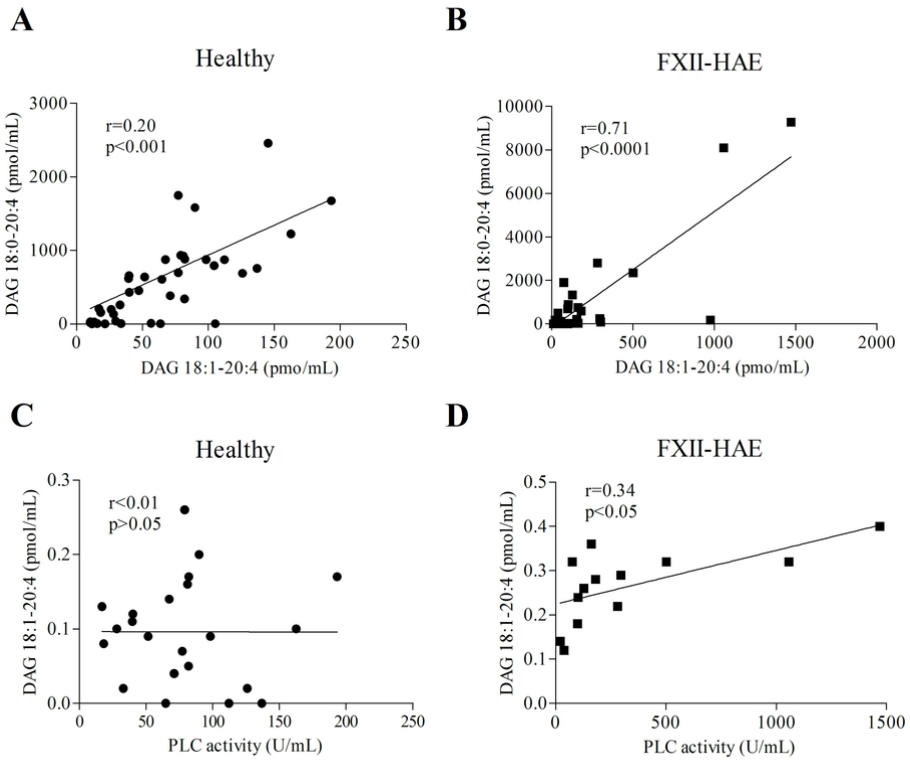

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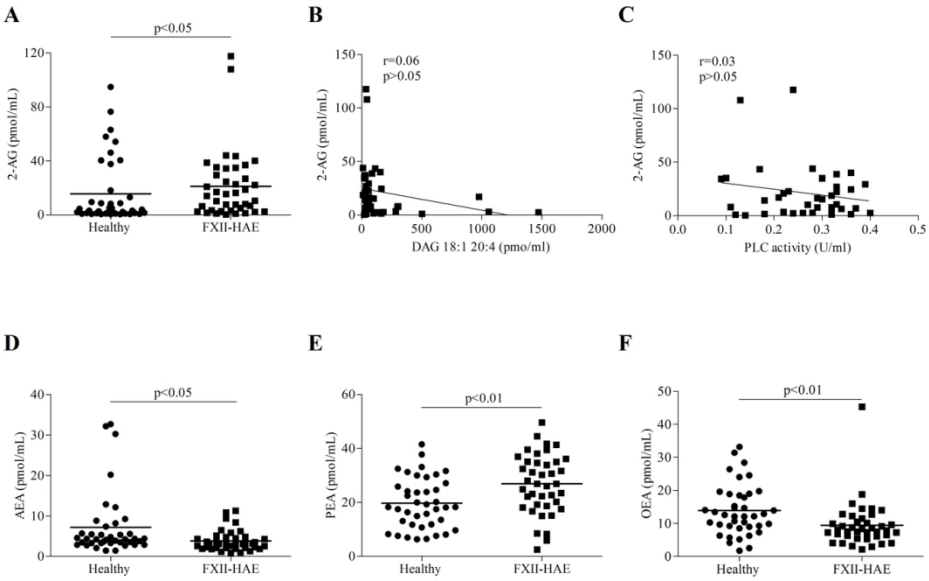

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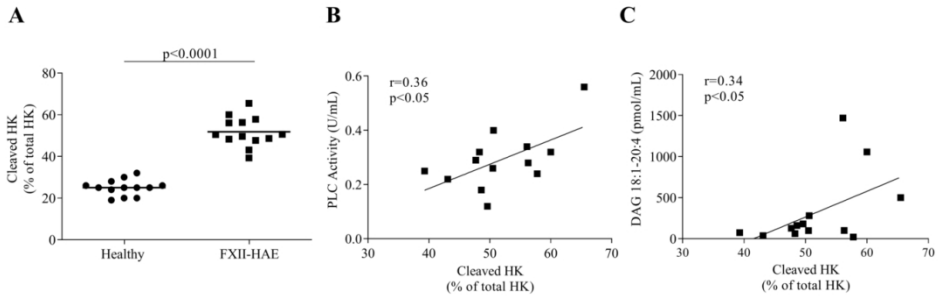

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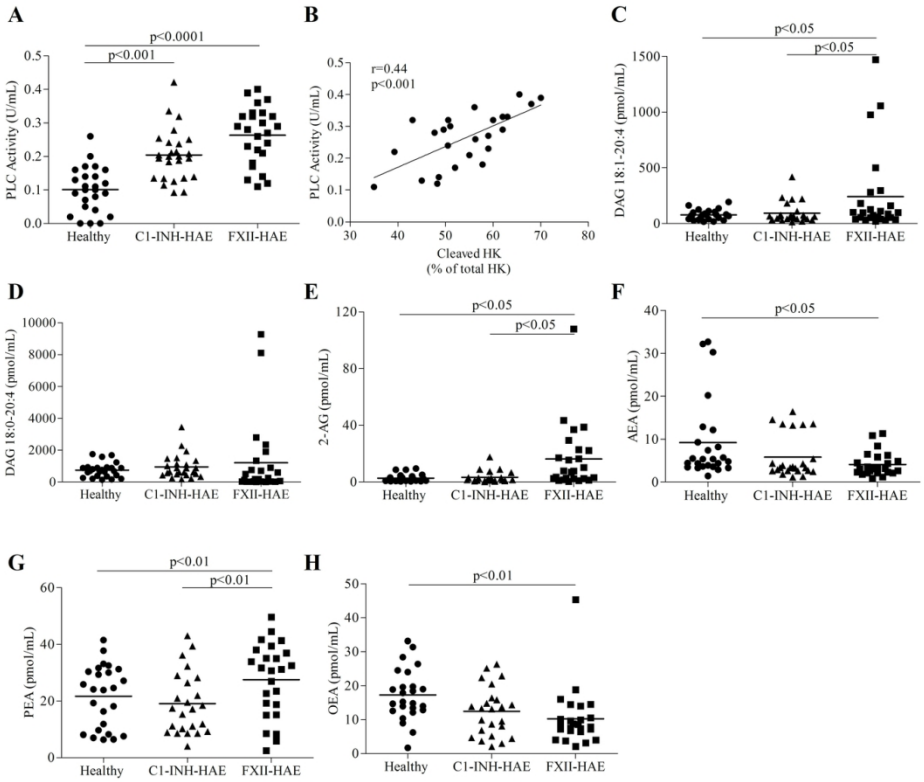

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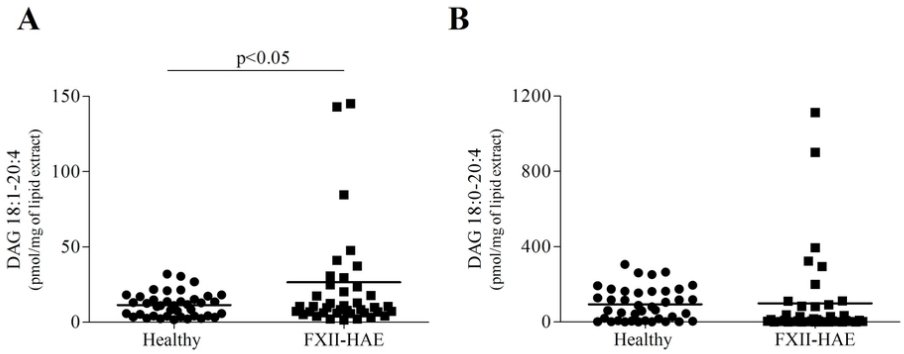

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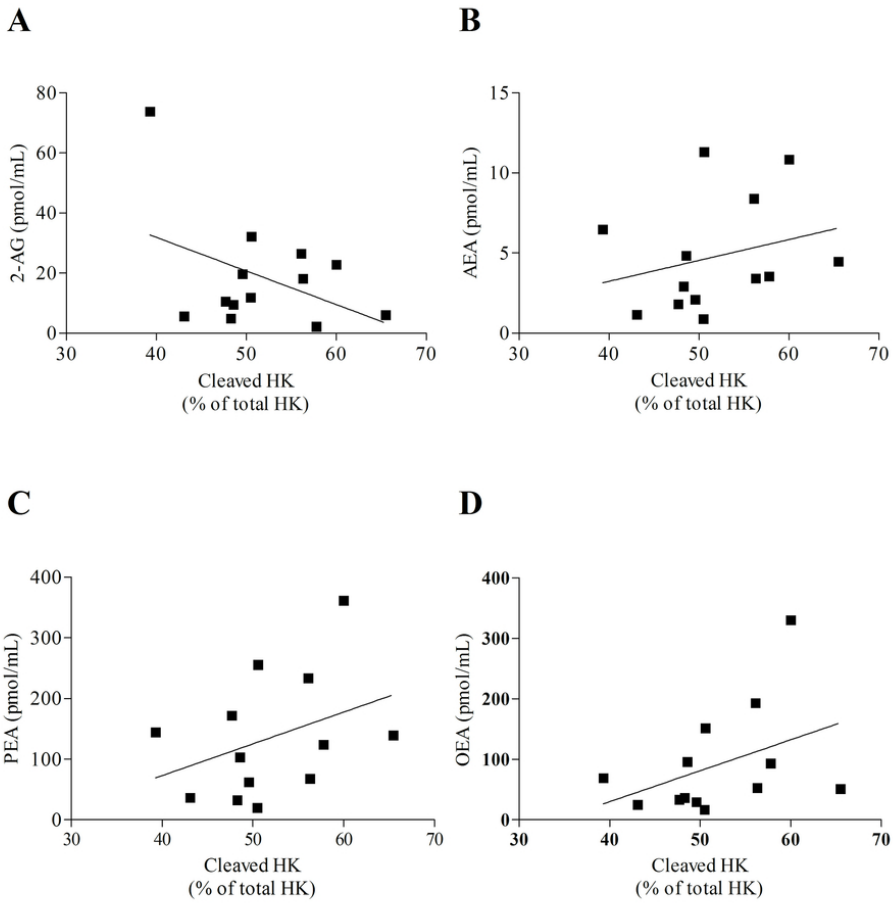

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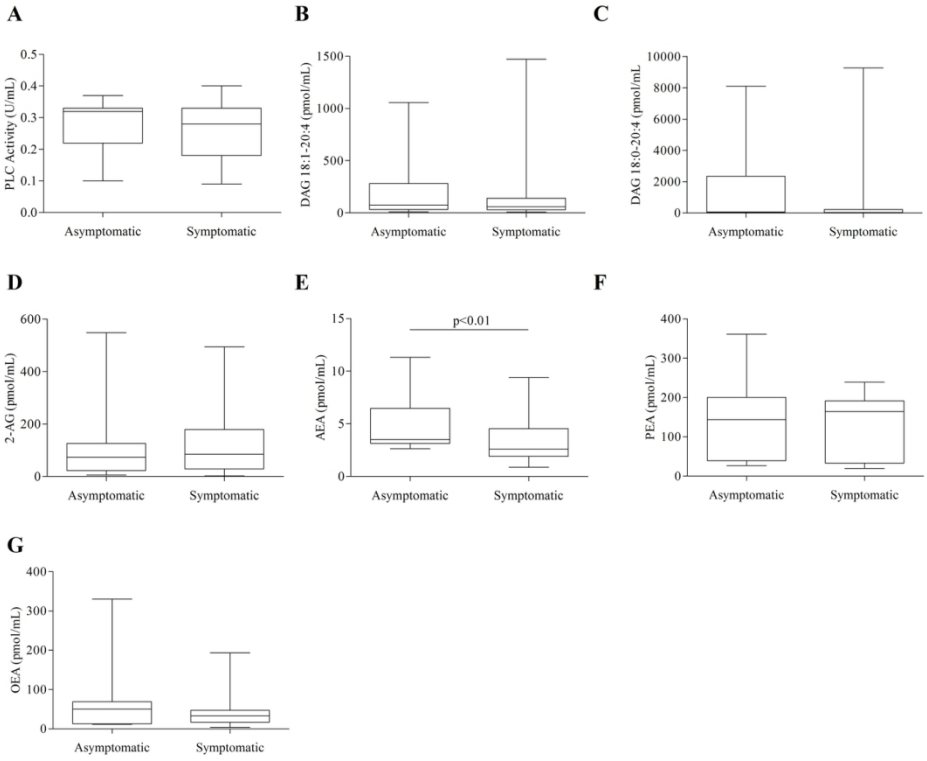

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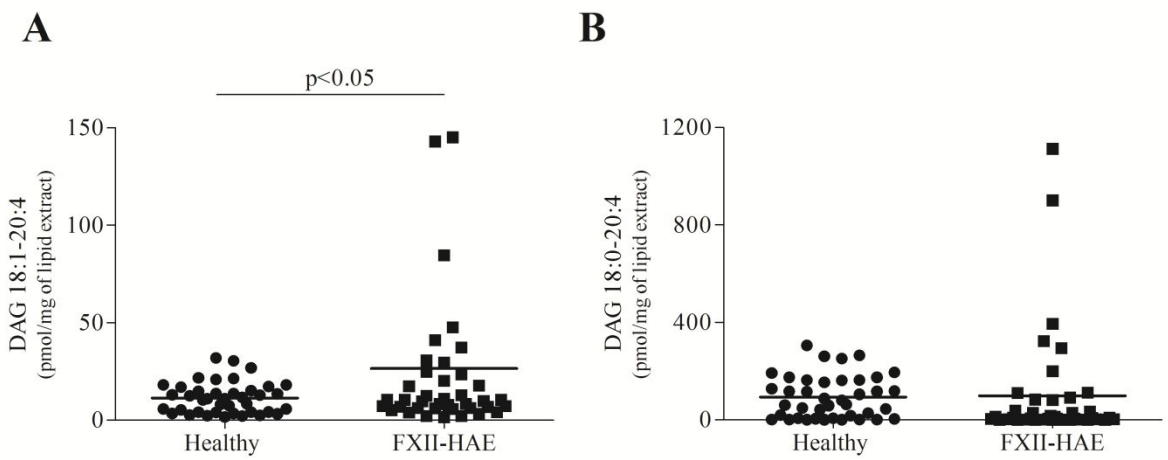
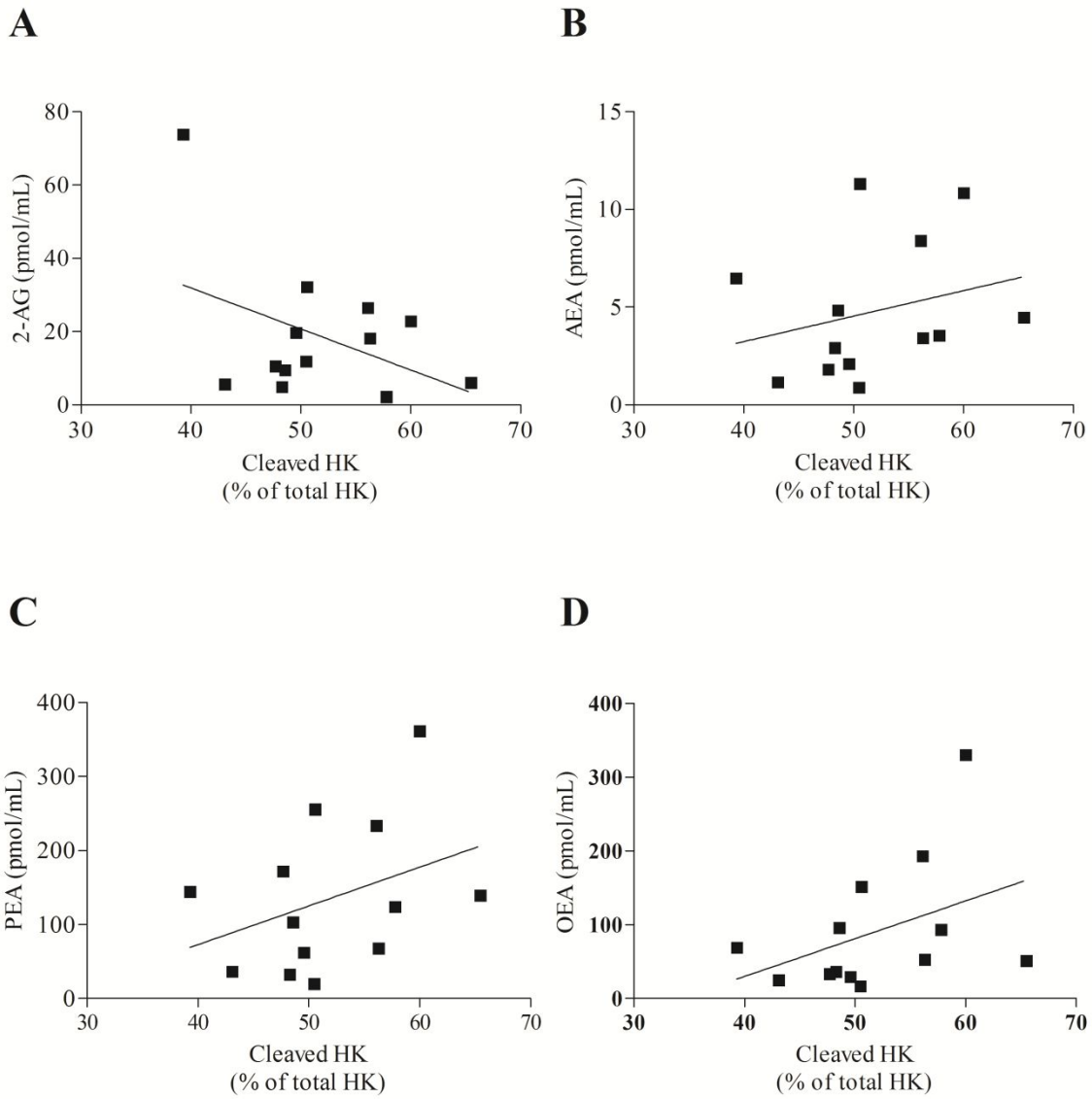
Supplementary Tab. 1 Demographic and clinical features of the patients with C1-INH-HAE, FXII-HAE and Healthy donors

Demographic and clinical features	Healthy (n=26)	C1-INH-HAE (n=26)	FXII-HAE (n=26)
Age-years (range)	42.6 (27-70)	42.6 (27-70)	42.6 (27-70)
Females – number (%)	21 (80.76%)	21 (80.76%)	21 (80.76%)
Caucasian patients	100%	100%	100%
Symptomatic	-	26 (100%)	22 (84.61)

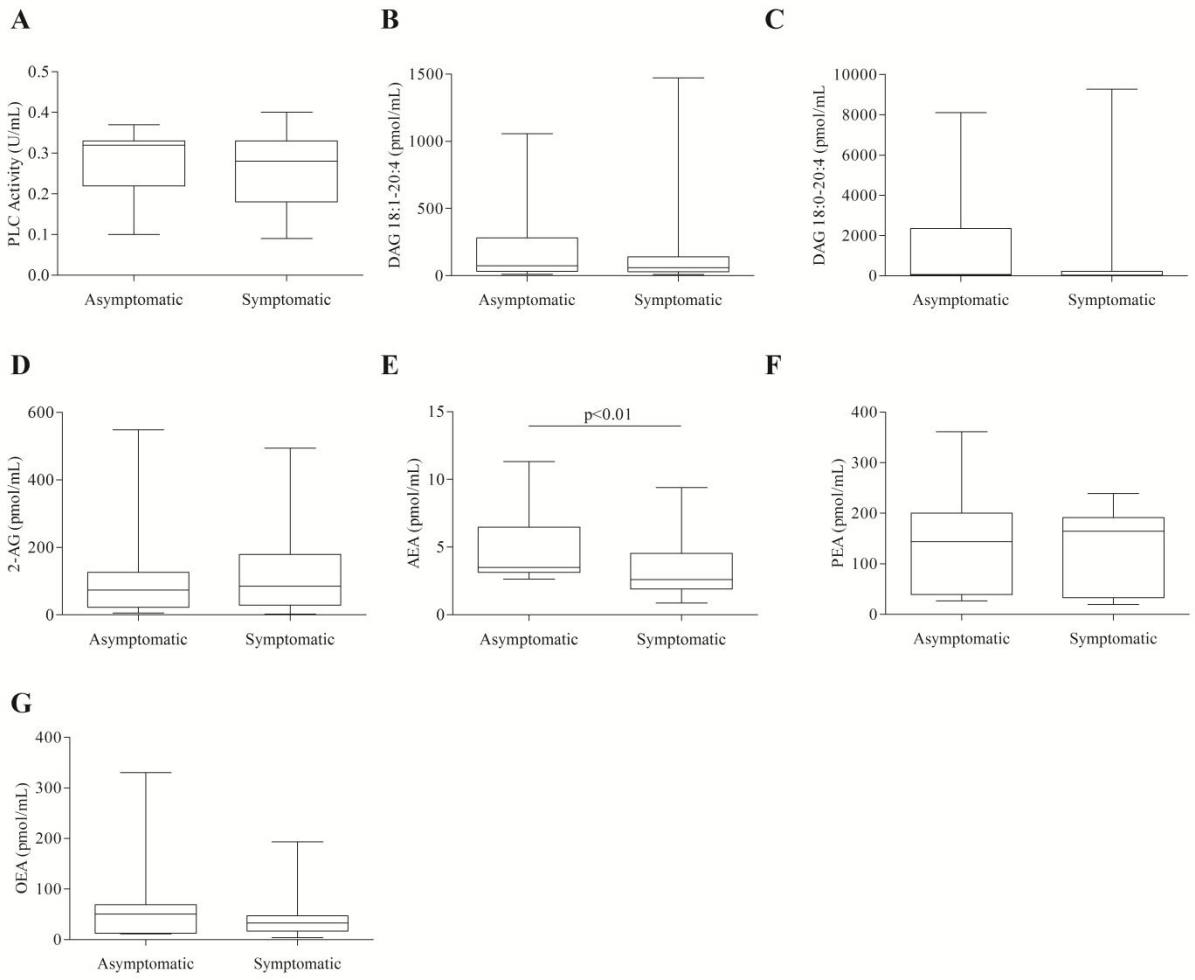


Supplementary Figure 1. DAG 18:1-20:4 and 18:0-20:4 concentrations expressed as pmol/mg of lipid extract in plasma of patients with FXII-HAE and healthy controls.

Plasma activity of PLC (A), DAG 18:1-20:4 (B) and 18:0-20:4 (C) concentration in 40 controls (Healthy) and in 40 patients with FXII-HAE in remission was determined by EIA and LC-MS-MS. Horizontal bars depict the median value. A p value ≤ 0.05 was considered statistically significant.

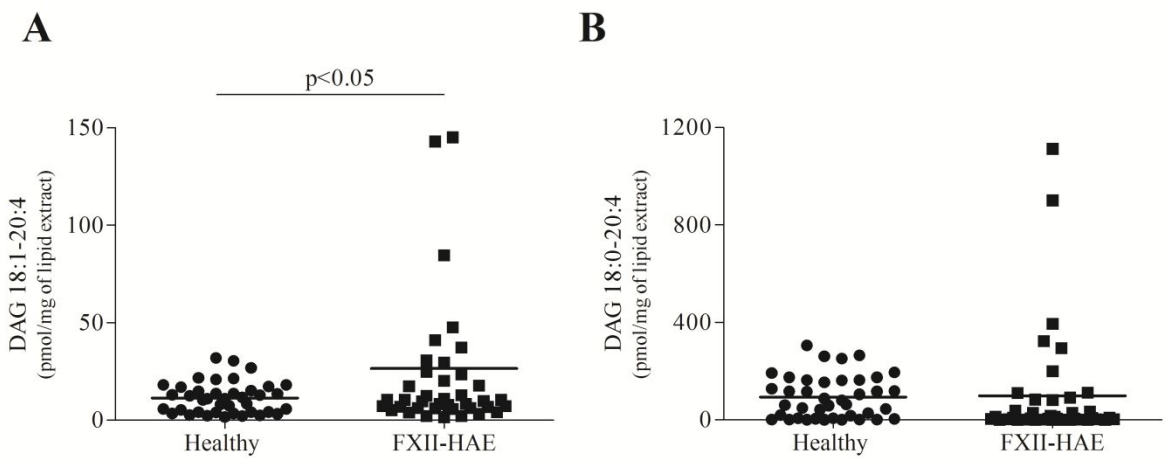


Supplementary Figure 2. Correlation among 2-AG, AEA, PEA, OEA and cHK in plasma of patients with FXII-HAE. Correlation between two variables: 2-AG and cHK (A), AEA and cHK (B), PEA and cHK (C), OEA and cHK (D) in 13 FXII-HAE patients were assessed by Spearman's correlation analysis and reported as coefficient of correlation (r). p value ≤ 0.05 was considered statistically significant.



Supplementary Figure 3. PLC, DAGs, eCBs and NAEs in plasma of asymptomatic and symptomatic FXII-HAE patients.

PLC (A), DAG 18:1-20:4 (B), DAG 18:0-20:4 (C), 2-AG (D), AEA (E), PEA (F), and OEA (G) were determined in 11 asymptomatic and 29 symptomatic FXII-HAE patients. Data are shown as the median (horizontal black line), the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 11 asymptomatic and 29 symptomatic FXII-HAE patients. **p* value ≤0.05 *vs.* asymptomatic.



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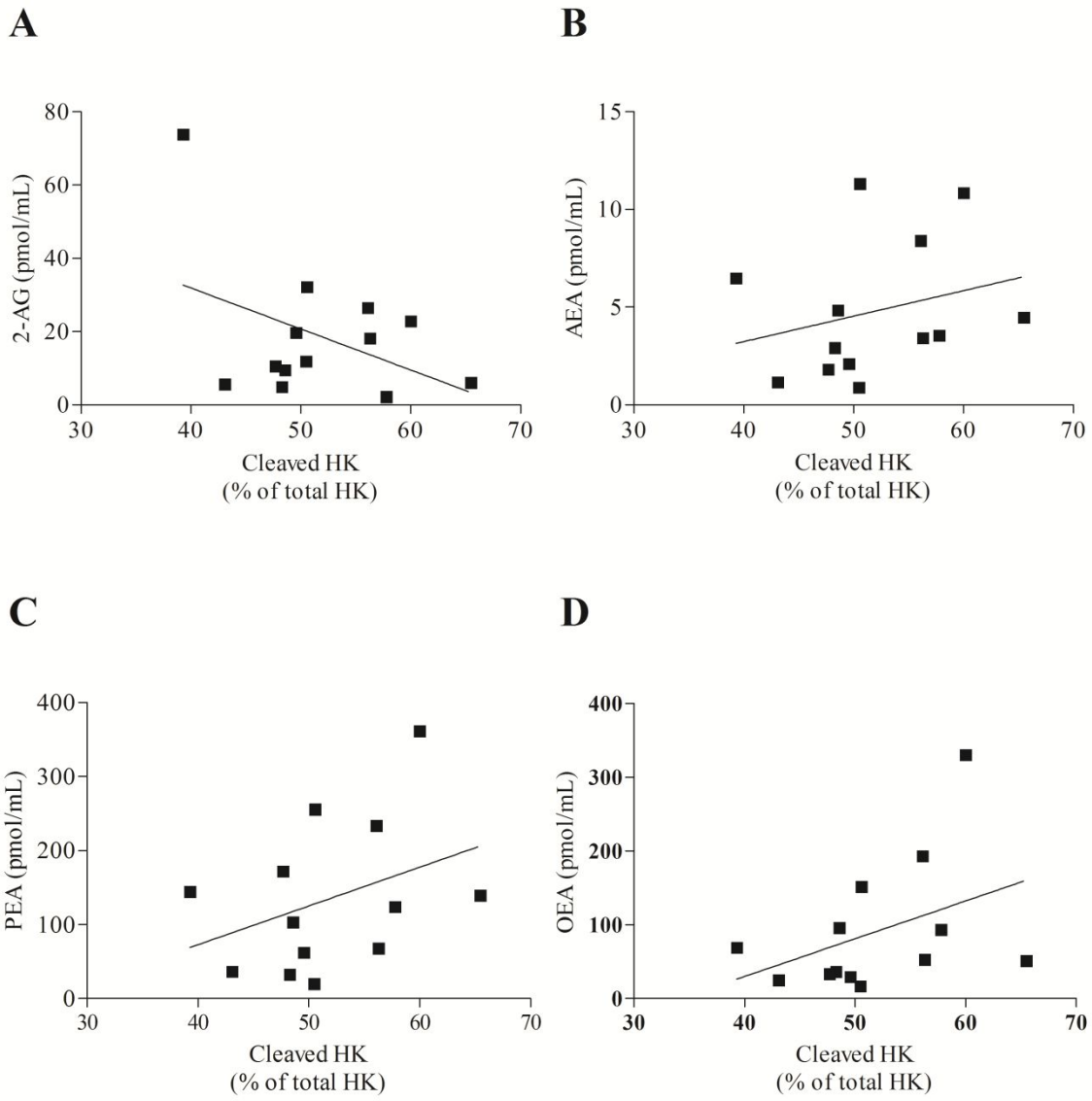
Plasma activity of PLC (A), DAG 18:1-20:4 (BA) and 18:0-20:4 (CB) concentration in 40 controls (Healthy) and in 40 patients with FXII-HAE in remission was determined by EIA and LC-MS-MS. Horizontal bars depict the median value. A p-value ≤ 0.05 was considered statistically significant.



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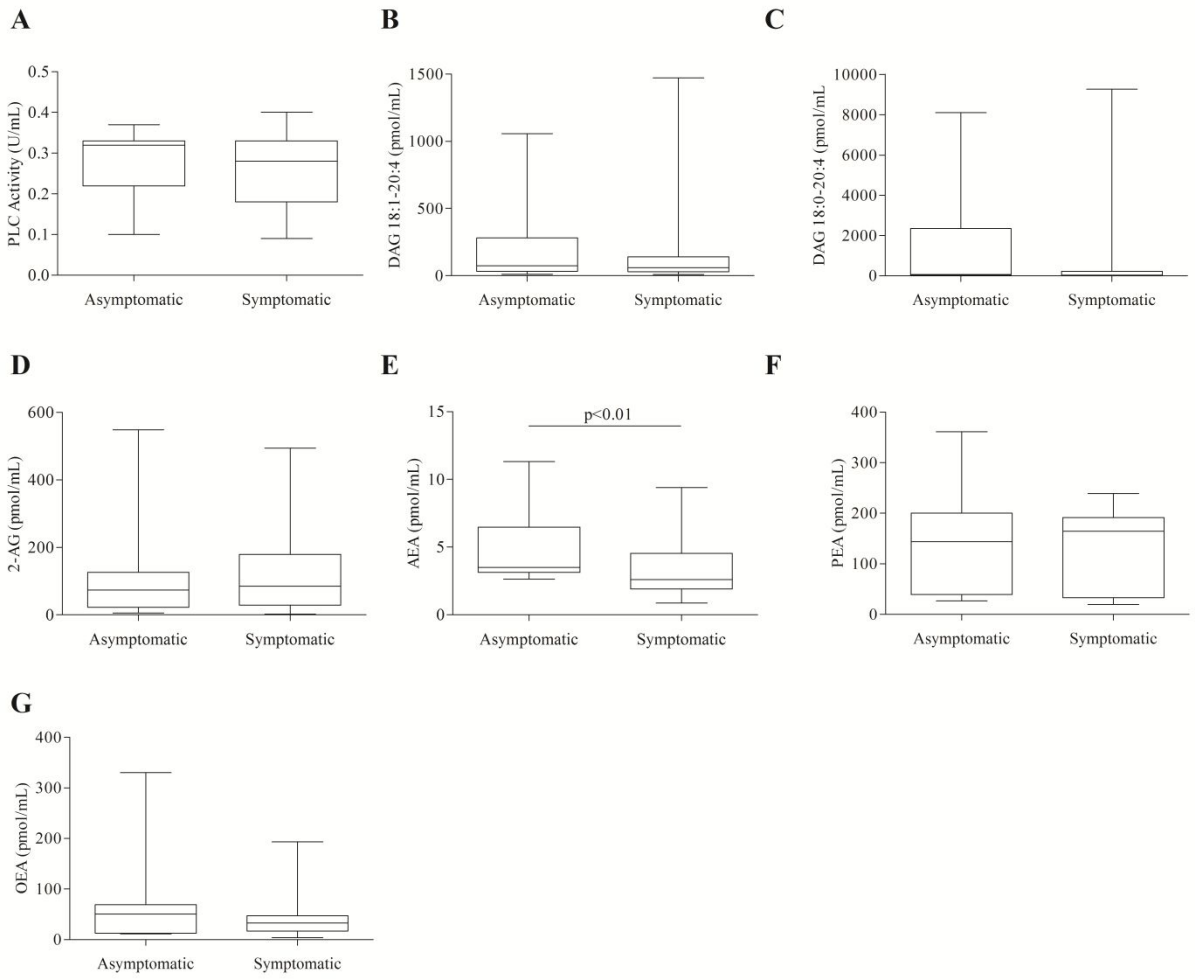

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