1 **Cold-induced urticarial autoinflammatory syndrome related to factor XII activation**

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Abstract

Hereditary autoinflammatory diseases are caused by gene mutations of the innate immune pathway, e.g. nucleotide receptor protein 3 (NLRP3). Here, we report a four-generation family with cold-induced urticarial rash, arthralgia, chills, headache and malaise associated with an autosomal-dominant inheritance. Genetic studies identify a substitution mutation in gene *F12* (*T859A*, resulting in p.*W268R*) which encodes coagulation factor XII (FXII). Functional analysis reveals enhanced autocatalytic cleavage of the mutated protein and spontaneous FXII activation in patient plasma and in supernatant of transfected HEK293 cells expressing recombinant W268R-mutated proteins. Furthermore, we observe reduced plasma prekallikrein, cleaved high molecular weight kininogen and elevated plasma bradykinin. Neutrophils are identified as a local source of FXII. Interleukin-1ß (IL-1ß) is upregulated in lesional skin and mononuclear donor cells exposed to recombinant mutant proteins. Treatment with icatibant (bradykinin-B2-antagonist) or anakinra (interleukin-1-antagonist) reduces disease activity in patients. In conclusion, our findings provide a link between contact system activation and cytokine-mediated inflammation.

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Introduction

Systemic autoinflammatory diseases (SAIDs) are characterized by self-directed inflammation that is mediated via activation of innate immune pathways. The majority of SAIDs are rare and disabling disorders with significant morbidity and quality of life impairment. Within the last 15 years, the molecular basis of several hereditary SAIDs was uncovered by using novel high throughput sequencing techniques. Many of the affected genes were shown to involve molecules associated with inflammasome signaling. Inflammasomes are intracellular multiprotein complexes, which assemble upon recognition of diverse pathogen- and danger-associated molecular patterns (PAMPs/DAMPs) leading to activation of caspase-1 to convert pro-interleukin-1ß (pro-IL-1ß) and pro-IL-18 into their active forms. Consequently, cytokine-targeted therapies, in particular directed against IL-1ß and its receptor, largely improved the treatment of SAID patients.

Cryopyrin-associated periodic syndrome (CAPS) is one of the best-studied hereditary SAIDs. Its hallmark feature is recurrent urticarial rash combined with systemic symptoms such as fever episodes, arthropathy, uveitis, headaches and malaise. Cold temperatures represent a major trigger of the disease. CAPS is caused by a heterozygous mutation in the gene encoding for nucleotide binding like receptor protein 3 (NLRP3 or cryopyrin).[1] The *NLRP3* mutation in CAPS results in an over-activated inflammasome followed by IL-1ß-mediated inflammation. A diagnosis of CAPS is often delayed and only eventually made by excluding other immune-mediated disorders, such as infections, immunodeficiencies, autoimmune and neoplastic diseases. Previously, up to 40% of cases with typical CAPS phenotype were

90   reported to lack disease-causing *NLRP3* mutations suggesting the existence of other, as of

91   yet unknown genetic variants.[2] The increased use of sequencing techniques identified a

92   number of somatic CAPS mutations in patients tested negative by conventional Sanger

93   sequencing (depending on the study and phenotype in 12-69% of individuals).[3,4] Besides

94   CAPS, few other genetic defects were found including mutations in *NLRP12, NLRC4* and

95   *PLCG2*. All of them present with early onset urticarial rash triggered by cold and an

96   autoinflammatory phenotype.[5-9]

97   Here, we describe an autosomal dominant disease characterized by cold-induced urticarial

98   rash and systemic inflammation. The disease is associated with a mutation in the human

99   coagulation Factor 12 gene (*F12*), and we propose the term FXII-associated cold

100   autoinflammatory syndrome (FACAS) to denote this disease. Functional studies show

101   autocatalytic cleavage of FXII and profound activation of the contact system linked to IL-1ß-

102   mediated inflammation.

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104   **Results**

105   *Clinical phenotype and disease manifestation*

106   Four family members of a four-generation family of European ancestry presented with cold-

107   induced urticarial rash that started within the first weeks after birth (**Table 1; Figure 1a**). The

108   non-pruritic burning rash occurred on a daily basis at the extremities, face and trunk and was

109   not accompanied by angioedema. Wheal development typically appeared within 10-30

110   minutes after whole-body exposure to cold temperatures (< 15° - 20°C) and stayed for one to

111   several hours after rewarming. The urticarial rash was exacerbated by windy and humid

112   weather. Also, wheals were induced by sweating and consecutive evaporative cooling. Direct

113   localized skin contact with cold fluids or objects, however, did not cause any symptoms. The

114   skin symptoms associated with signs of systemic inflammation including chills, joint pain,

115   headache, fatigue and subfebrile evening temperatures. All patients had perennial symptoms

116   that deteriorated during the cold season and frequently lead to absence from school and

117   work. Over the course of their disease, patients reported worsening of systemic

118   manifestations, e.g. joint pain, fatigue and chills, resulting in a more severe phenotype in

119   adult patients. The clinical signs were combined with mild to moderate increases in serum

120   levels of the inflammation markers amyloid A (SAA) and S100 A12/S100 A8/9, but not C-

121   reactive protein (CRP). Other routine laboratory markers (e.g., liver and kidney function,

122   blood count, coagulation) were unremarkable. Histopathology of lesional skin following cold

123   exposure revealed moderate dermal edema and perivascular infiltrates composed of

124   macrophages and few neutrophils (**Figure 1b**). In contrast to acquired cold urticaria, affected

125   individuals presented with generalized urticarial rash upon cold exposure and tested negative

126   in the ice cube skin provocation test. Cold water bath provocation of the forearm (15°C for 10

127   min) did also not cause any wheals or angioedema. In addition, all patients reported

128   complete unresponsiveness to oral antihistamines at standard or higher than standard doses

129   (please refer to **Supplementary Table 1** for a comparison of FACAS with other cold-induced

130   urticarial disorders). A therapeutic trial with IL-1 receptor antagonist anakinra in patients #1

131   and #2 resulted only in limited (#2) to moderate improvement (#1) of clinical symptoms.

132   However, continuous anti-IL-1 treatment could not be maintained due to severe injection site

133   reactions.

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135   *Genetic analysis*

136   Based on the clinical phenotype resembling CAPS, Sanger sequencing of *NLRP3* and

137   *NLRP12* was initially performed, but failed to identify plausible pathogenic mutations in the

138   index patient (#1, **Figure 1c**). The following whole exome sequencing prioritised the

139   assessment of genes that were previously associated with a skin phenotype of urticaria or

140   urticaria-like symptoms as reported by the Human Gene Mutation Database (Release

141   2015.4).[10] These were *TBXA2R*, *CEP68*, *F12*, *SERPING1*, *NLRP3*, *NLRP12*, *KIT*, *IL18*,

142   *HNMT, FCER1α*, *NLRC4*, *ADGRE2* and *PLCG2*. Sequencing data was generated for three

143   affected family members across three generations (#2, #5, #7, **Figure 1c**) and filtered to

144   identify a single candidate variant, a heterozygous substitution mutation in exon 9 of *F12*

145 (**Figure 1d**), the gene encoding for the human coagulation factor XII

146 (NM_000505:*c.T859A:p.W268R*). The observed variant is not listed in the Genome

147 Aggregation Consortium (gnomAD) database listing ~140,000 individuals.[11] *In silico*

148 predictors of variant pathogenicity concordantly inferred deleterious impact (GERP = 5.01;

149 SIFT = 0.0; PolyPhen2 = 1.0). Sanger sequencing of the available family members confirmed

150 consistent segregation of this mutation with the prior annotation of disease status (**Figure**

151 **1c,d**).

152 FXII exerts diverse functions in coagulation, fibrinolysis, complement activation and the

153 kallikrein-kinin system. Mutations in *F12* are linked to hereditary angioedema with normal C1

154 inhibitor (FXII-HAE).[12] FXII-HAE is characterized by acute attacks of localized tissue

155 swellings that are mediated by the vasoactive peptide bradykinin, which is produced by the

156 plasma kallikrein-kinin system. Known FXII-HAE mutations are primarily located in the

157 proline-rich region on exon 9.[13,14] In contrast, the W268R variant lies within the triple-looped

158 kringle domain of FXII.

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160 *Functional analyses*

161 Markers of coagulation (activated partial thromboplastin time [aPTT], fibrinogen,

162 plasminogen, FXI activity, D-dimers, INR) and complement activation (C3, C4, C1-esterase

163 inhibitor [C1-INH] concentration and function) were normal in all affected family members.

164 To test, if the FXII-*p.W268R* substitution in our patients leads to cleavage and/or activation of

165 FXII associated with contact system activation and subsequent generation of bradykinin, we

166 first assessed FXII fragmentation and activity. For its activation, FXII needs to be cleaved

167 into a two-chain molecule composed of a heavy and a light chain linked by a disulfide bond.[15]

168 In addition to the unprocessed FXII (78 kDa), molecular analyses revealed an increased

169 presence of the 50 kDa band (reducing) in the FXII immunoblot from plasma of affected

170 family members which was hardly detectable in healthy control family members and an FXII-

171 HAE control (**Figure 2a**). This band resembled the heavy chain of active FXII (FXIIa),

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172 suggesting partial fragmentation and activation of FXII in affected patients. Accordingly,

173 recombinant FXII-*p.W268R* (rFXII W268R) expressed in HEK293 cells also showed the

174 characteristic 50 kDa (III) band in the FXII immunoblot resembling the heavy chain. This

175 band is also present in purified αFXIIa but not found in unprocessed recombinant FXII (rFXII

176 wt) (**Figure 2b**). In addition to the heavy chain, the light chain at 25 kDa (IV) was found in

177 preparations of αFXIIa and rFXII W268R but not in rFXII wt. Treatment of rFXII W268R with

178 kaolin resulted in increased generation of the light chain fragment. Besides the heavy and

179 light chains at 50 kDa and 25 kDa respectively, we found in the rFXII W268R preparation a

180 third fragment with an apparent molecular weight of 60 kDa (reducing) that was absent in

181 the αFXIIa preparation (**Figure 2a,b**). Mass spectrometry analysis of excised gel fragments

182 confirmed the autoproteolytic cleavage of rFXII W268R at residues AA353/354 which yields

183 in FXIIa. In addition, analysis of the 60 kDa fragment revealed cleavage of rFXII W268R after

184 AA447, which is part of the protease domain and facilitated by kaolin treatment (**Figure 2c**).

185 In line with these observations which suggest autoactivation of FXII W268R, we found high

186 spontaneous activity of FXIIa in the plasma of affected family members (**Figure 2d**) as well

187 as in the supernatant of FXII W268R-expressing HEK293 cells but not in the plasma of

188 healthy family members or wt-FXII expressing HEK293 cells (**Figure 2d,e**). Interestingly,

189 overall plasma levels of FXII were comparable to healthy controls (**Supplementary Figure**

190 **1**), indicating that the *p.W268R* mutation only affects the probability of spontaneous FXII

191 activation, but not its expression.

192 We next assessed mediators of the kallikrein-kinin pathway. High FXIIa activity was

193 associated with pronounced plasma prekallikrein (PK) cleavage and kallikrein formation

194 (**Figure 3a)** similar to what is found in activated recombinant PK preparations

195 **(Supplementary Figure 2**). In line with these findings, we noticed reduced PK activity and

196 plasma levels in affected patients (**Figure 3b,c**). FXII-mediated contact system activation is

197 counterbalanced by C1-INH to prevent continuous kallikrein and bradykinin formation. Thus,

198 our observation of increased FXIIa-C1INH and PK-C1-INH complexes in FACAS plasma

199 reflected an imbalanced and constitutively active contact system (**Figure 3d,e**). Kallikrein

cleaves high molecular weight kininogen (HMWK) to release bradykinin, a potent but short-lived vasoactive mediator acting on bradykinin B2 receptors. Since bradykinin becomes rapidly degraded by kininases,[16] cleaved HMWK (cHMWK) is used as a surrogate marker of plasma bradykinin formation.[17] Interestingly, we found complete degradation of HMWK in the immunoblot of all tested FACAS patients (**Figure 3f**). Correspondingly, the activity of cHMWK was clearly upregulated in patients vs. controls (**Figure 3g**). Of interest, bradykinin plasma levels which were available from two FACAS patients revealed higher values compared to healthy controls (**Figure 3h**).

To investigate the possibility of local FXII production and activation at sites of inflammation, we evaluated lesional skin of a FACAS patient following cold exposure. Immunohistochemistry revealed FXII expression by intra- and perivascular neutrophils (but not macrophages; **Supplementary Figure 3**) as demonstrated by cytoplasmatic co-staining with antibodies for myeloperoxidase (MPO) and α-FXIIa heavy chain (**Figure 4a**). Moreover, we found FXII expression in single peripheral blood neutrophils purified from an affected patient but not in healthy controls (**Figure 4b**). Together, these findings could point towards local FXII production by neutrophils in the skin of FACAS patients. As monocytes and tissue macrophages are known to recruit neutrophils via IL-8 secretion,[18,19] we hypothesized that contact system mediators might induce the attraction of neutrophils. Accordingly, we found dose-dependent IL-8 release from monocytes treated with increasing doses of bradykinin (**Figure 4c**).

FXIIa and bradykinin were previously shown to induce IL-1ß secretion from human monocytes and macrophages.[20,21] Assuming that FXIIa and/or consecutive mediators like bradykinin affect the IL-1ß-related phenotype in FACAS, we investigated IL-1ß responses in patient samples. Basal IL-1ß secretion from patient PBMCs was negligible and comparable to healthy control PBMCs (**Supplementary Figure 4**). However, exposure of healthy donor PBMCs to recombinant FXII proteins (wild-type and W268R) suggested an IL-1ß priming effect of the FXII W268R mutant protein but not wild-type protein when co-stimulated with the

227 NLRP3 activator ATP (without LPS) (**Figure 5a**). To investigate whether the IL-1ß priming

228 effect was mediated via FXIIa activity, we assessed the potential of recombinant FXII W268R

229 with an S544A inhibitory mutation[22] (rFXII W268R-S544A) and αFXIIa to induce IL-1ß

230 release in PBMCs. Moreover, we blocked protease activity with the small peptide-based

231 inhibitor PPACK (Phe-Pro-Arg-chloro-methylketone). Remarkably, the incapacitated S544A

232 mutant protein showed a similar IL-1ß priming effect as the active rFXII W268R (**Figure 5b**).

233 In contrast, the priming effect on IL-1ß synthesis was absent in PBMCs treated with αFXIIa

234 (**Figure 5c**). Also, in LPS-primed and ATP-activated PBMCs, addition of FXIIa or FXIIa-

235 PPACK did not induce significant changes on IL-1ß release (**Figure 5c**). Together, these

236 results demonstrate that the priming effect on IL-1ß synthesis is not linked to FXIIa activity.

237 When analyzing biopsies from lesional FACAS skin, we observed pronounced IL-1ß

238 expression (**Figure 5d,e**). Positive IL-1ß staining co-localized with perivascular neutrophils

239 and macrophages suggesting these cells as the primary source for IL-1ß in the skin.

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241 *Clinical efficacy of bradykinin B2 receptor antagonism*

242 Based on the collected evidence for excessive contact system activation in FACAS, we

243 initiated a short treatment course over four consecutive days with the bradykinin B2 receptor

244 antagonist icatibant in the index patient (patient #1). Individual symptoms were assessed by

245 a patient-reported standardized daily health assessment form that was adapted from a

246 validated tool for CAPS.[23] Within 30 minutes after the first administration of 30 mg icatibant,

247 the generalized urticarial rash and systemic symptoms improved. The maximum effect

248 (absence of rash and headache, largely improved fatigue) occurred after 90 minutes and

249 lasted for up to 12 hours. Icatibant treatment was initiated during the winter and tolerated

250 well. It completely abrogated skin and systemic symptoms while staying outside for several

251 hours at temperatures below 0°C. Only arthralgia was still mildly present. Complete relapse

252 of symptoms occurred within 24h after drug withdrawal (**Figure 6**).

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Discussion

We report a dominantly inherited cold-induced urticarial autoinflammatory disease in a 4-generation family that is associated with a *c.T859A* mutation in *F12* leading to an amino acid exchange of tryptophan by an arginine (*p.W268R*). Functional analyses of the mutant protein revealed high susceptibility to autocatalytic cleavage after residue AA353, which is known to be associated with FXIIa formation. This is paralleled by constitutive activation of the *p.W268R* variant of FXII resulting in an overactive contact system and release of potent inflammatory cytokines such as IL-1ß. The relevance of bradykinin as critical mediator is underlined by the immediate clinical response to treatment with the bradykinin B2 receptor antagonist icatibant. Skin and systemic symptoms associated with IL-1ß-mediated inflammation in FACAS are reminiscent of the clinical phenotype of CAPS. Its underlying mechanisms, however, are different and share signaling cascades of kallikrein-kinin activation with HAE.

FXII is primarily produced and secreted by hepatocytes. It circulates as a single-chain inactive zymogen in the plasma and converts into FXIIa (composed of an N-terminal heavy chain and a C-terminal light chain) upon contact with negatively charged surfaces such as polyphosphates released by platelets or by limited proteolysis.[15] In the pathogenesis of FXII-HAE, defective glycosylation augments contact-mediated FXII autoactivation, which enhances bradykinin formation via the kallikrein-kinin system.[24] Our data, showing partially cleaved and active FXII in patient plasma as well as enzymatic activity of recombinant W268R in cell culture supernatants, suggest spontaneous, intracellular conversion of FXII into FXIIa in FACAS. In contrast, spontaneous FXII cleavage was absent in FXII-HAE plasma, which is in line with previous reports showing the need for a plasmatic trigger to cleave and activate FXII in HAE.[25]

The novel mutation induces an exchange of tryptophan to arginine (*W268R*) inside the kringle domain of FXII. Kringles are common in members of the coagulation cascade and are thought to play essential roles in binding target molecules/structures, such as

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281 membranes, other proteins or phospholipids, and in the regulation of proteolytic activity.[26,27]

282 Our observation of fragmented rFXII-*W268R* that is cleaved after residue AA353 argues for

283 increased autoactivation and may explain the high spontaneous FXIIa activity observed in

284 plasma and supernatant of HEK293 cells transfected with rFXII-W268R. These findings differ

285 from recent studies in FXII-HAE with p.*T309R* mutation. This variant was demonstrated to be

286 enzymatically truncated after residue AA309 resulting in the loss of the heavy chain fragment

287 and dysregulated PK activation.[28] Our data do not support truncation of *W268R* after residue

288 AA268. In contrast, we observed additional cleavage after AA447 in the recombinant protein.

289 However, this was not detected in plasma of the affected patients suggesting that this might

290 be the result of artificial expression in HEK293 cells. From our data we conclude that high

291 susceptibility to cleavage at the regular cleavage site at AA353/354 leads to continuously

292 increased FXIIa activity. In addition, the exchange of the aromatic and lipophilic tryptophan to

293 the hydrophilic and, at physiological pH, positively charged arginine may foster binding of

294 mutant FXII to negatively charged surfaces, thereby further enhancing its activation state

295 leading to an exhaustion of the contact system with fully cleaved PK and HMWK and

296 constantly raised bradykinin levels in the patient plasma.

297 Neutrophil-dominated dermal infiltrates are known from urticarial autoinflammatory diseases

298 Schnitzler's syndrome and CAPS.[29] These are classified as neutrophilic urticarial dermatosis

299 as opposed to the rather sparse lympho-histiocytic infiltrates in lesional skin of common

300 urticaria. Their role, however, remains ill-defined. Interestingly, peripheral blood neutrophils

301 were previously shown to produce zymogen FXII, which functionally differs from hepatic FXII

302 and contributes to neutrophil trafficking via cell adhesion, migration and release of

303 extracellular DNA at sites of inflammation.[30] Conceivably, the generalized urticarial rash in

304 FACAS could be mediated via neutrophil-derived and pre-activated FXII at the sites of

305 inflammation as suggested by the cytoplasmatic expression of αFXIIa in skin and peripheral

306 blood neutrophils. IL-8 is known to induce neutrophil recruitment.[19] Supported by our data,

307 plasma-derived BK may induce IL-8 production by skin macrophages to recruit neutrophils

308 (**Figure 7**). We hypothesize that upregulated FXII expression in FACAS neutrophils may be

309   mediated via a paracrine feedback loop involving contact system components such as

310   plasma kallikrein and HMWK. These were both shown to activate (and in case of HMWK also

311   bind to) neutrophils.[31,32] Thus, the high degree of contact system activation could be the

312   reason why FXII expression was restricted to FACAS and not observed in healthy control

313   neutrophils.

314   The sensitivity to cold as a trigger and amplifier of clinical symptoms in FACAS is known from

315   autoinflammatory diseases such as CAPS. Here, cold was proposed to escalate constitutive

316   inflammasome activity via increased production of reactive oxygen species induced by

317   cellular stress to temperature shifts.[33] In FACAS, however, inflammasomes of mononuclear

318   cells are not constitutively activated (**Supplementary Figure 4**), suggesting alternative

319   mechanisms. Of interest, C1-INH activity was previously reported to be inhibited by cold as

320   shown by complete plasma HMWK cleavage at 0°C *in vitro*.[34] We assume that cold-induced

321   decreases in tissue C1-INH activity may contribute to an imbalanced contact system in

322   FACAS. Temperatures above a certain threshold (clinically around 20°C) may protect from

323   inflammatory symptoms because of adequate local C1-INH activity that counterbalances

324   kallikrein activation. Based on this hypothesis, lower temperatures will reduce C1-INH activity

325   leading to spontaneous but locally restricted activation of the kallikrein-kinin pathway and

326   bradykinin production in the tissue, sufficient for wheal induction but not angioedema.

327   Bradykinin drives the inflammation in experimental models of osteoarthritis and

328   neuroinflammation via upregulation of cytokines such as IL-1ß.[35,36] Vice versa, IL-1ß is

329   known to augment the accumulation of bradykinin via up-regulation of bradykinin receptors

330   [37,38] and activation of prekallikrein-HMWK complexes.[39] Also, IL-1ß downregulates

331   angiotensin converting enzyme (ACE) expression in endothelial cells, resulting in less

332   efficient bradykinin elimination.[40] Accordingly, elevated skin and systemic IL-1ß levels in

333   FACAS may amplify inflammatory responses via a positive feedback loop that induces

334   further bradykinin production. Moreover, bradykinin was shown to activate and degranulate

335   mast cells *in vitro* resulting in the release of heparin and polyphosphates, which may facilitate

336   the local production of bradykinin via FXII activation.[41,42] Also, skin mast cells contribute to IL-

337   1ß-mediated inflammation in urticarial autoinflammatory diseases,[43] and their abundant

338   expression and activation may promote the urticarial phenotype in FACAS patients (**Figure**

339   **7**).

340   The clinical response to treatment with the IL-1 receptor antagonist anakinra mirrors

341   the increased presence of IL-1ß in FACAS tissue similar to other urticarial autoinflammatory

342   diseases such as CAPS. Incomplete clinical improvement by anakinra may be explained by

343   continuous bradykinin generation via complex feedback loops and possibly further cytokines

344   such as TNFα, which are induced by bradykinin [44] and not inhibited by IL-1 blockade. Of

345   further interest, our findings suggest that the *p.W268R* variant can prime cells for IL-1ß

346   production via pattern recognition receptors without the need of an exogenous stimulus by

347   pathogen associated molecular patterns such as LPS. Surprisingly, the IL-1ß priming effect

348   is not linked to FXIIa activity as shown by the missing impact of αFXIIa on IL-1ß secretion.

349   Thus, we believe that conformational changes of FXII *p.W268R* result in the recognition as a

350   danger signal that serves as toll-like receptor agonist and inducer of IL-1ß release.

351   The prompt and striking clinical response to the bradykinin B2 receptor antagonist icatibant is

352   in line with its efficacy in HAE patients and strongly supports our model of a bradykinin-

353   mediated mechanism. Reasons for the persistence of mild arthralgia may be the short

354   treatment duration of icatibant or the fact that bradykinin effects on joint inflammation are

355   mediated via the B1 receptor rather than the B2.[35]

356   Limitations of our study include the small patient and sample sizes. Until now, there are no

357   other families or individuals known with FACAS. Hence, it will be important to screen patients

358   with similar phenotypes for the FXII *p.W268R* mutation in order to confirm our data. The

359   mechanism by which cold induces inflammation as well as the role of neutrophils in FACAS

360   need to be further evaluated. Also, the duration of treatment with anakinra and icatibant was

361   only short due to side effects and limited efficacy (anakinra) or limited availability (icatibant).

362   Data on long-term efficacy are still missing.

363   In conclusion, the pathomechanisms underlying this novel disease illustrate the critical role of

364   fragmented and pre-activated FXII in driving skin and systemic inflammation as well as the

365   close link between contact system activation and cytokine signaling. Therapeutic approaches

366   in FACAS should aim at continuous control of inflammation in order to prevent long-term

367   sequelae such as amyloidosis. Based on the short half-life of icatibant of few hours, inhibitors

368   of contact activation including kallikrein antagonists and FXII antibodies are promising drug

369   candidates of interest.

370

371   **Methods**

372   *Patients*

373   A total of eight family members, four of them affected and four non-affected, were analyzed.

374   All of them provided written informed consent. This included the publication of individual

375   data. For affected children, parents provided written informed consent respectively. The

376   authors affirm that human research participants provided informed consent for publication of

377   the images in Figures 1a and 6a. The study was approved by the local Charité Ethics

378   Committee, Charité - Universitätsmedizin Berlin, Germany.

379

380   *Genetic studies*

381   Genomic DNA was isolated from saliva samples of eight family members, and whole exome

382   sequencing (SureSelect human all exon v5.0, Agilent, Santa Clara, California) of three

383   affected family members across three generations was performed and analysed as

384   described before in greater detail.[45] Briefly, genetic variants in the candidate genes were

385   filtered to be present in all three sequenced individuals (in any zygosity) and to occur at < 5%

386   in 1000 Genomes database. DNA was available for follow-up Sanger sequencing from seven

387   family members including all four affected individuals to determine consistency of variant

388   segregation with disease. DNA exome capture was performed using the SureSelect human

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389   all exon v5 kit according to the manufacturer's protocol and the library sequenced on the

390   HiSeq 2000 platform (Illumina, San Diego, California), generating data sufficient to provide

391   >50X coverage across the exome. Read data were aligned using Novoalign v2.08 and

392   duplicate reads marked using Picard v1.97. Variant calling was performed using Samtools

393   v0.1.19 and variants annotated using Annovar (23rd August 2013 release) utilizing RefSeq

394   gene definitions. Candidate genes were identified in HGMD release 2015.4 using the search

395   term urticaria. Segregation analysis was performed on amplicons generated using primers

396   forwards 5'-CTAACCTCCCGGGGTCTG-3' and reverse 5'-CAGACCCCGGGAGGTTAG-3'

397   using standard techniques. Unidirectional Sanger sequencing was performed using the

398   forward primer.

399

400   *Blood collection*

401   Following standard procedures, 9 volumes of blood were collected in 1 volume of sodium

402   citrate, (3.2% w/v). Samples were centrifuged twice at 2000 x g for 10 min. Plasma from

403   FACAS-affected family members and from healthy donors was stored in aliquots at -80°C

404   until use. For PBMC stimulation assays, blood from FACAS-affected family members or

405   healthy control donors was sampled into lithium heparin tubes and processed as explained

406   below. Citrate and heparin tubes were from Greiner Bio One (Kremsmünster, Austria). For

407   plasma bradykinin level assessment, blood was sampled into SCAT-169-4.5/5 protease

408   inhibitor blood collection tubes (Haematologic Technologies, Essex Junction, Vermont),

409   centrifuged at 2000 x g for 10 min and stored as described above.

410

411   *Western Blot*

412   Frozen plasma of affected and healthy individuals was thawed on ice, diluted 1:10 (FXII),

413   1:20 (HMWK) or 1:40 (PK) in sample buffer (15.5% glycerol, 96.8 mM Tris-HCl, 3.1% SDS,

414   and 0.003% bromophenol blue, 25 mM DTT [Sigma-Aldrich, St. Louis, Missouri]), boiled for

415 5-10 min and briefly chilled on ice. Recombinant constructs were treated similarly.

416 Approximately 50-100 ng total protein of recombinant proteins or 5 µl of plasma sample were

417 loaded and separated on 10% Tris/glycine or Bolt 4-12% Bis-Tris Plus Gels (Merck-Millipore)

418 and separated at 200 V (running buffer 190 mM glycine, 25 mM Tris, 0.1% SDS) or 165 V

419 (MOPS running buffer [Merck-Millipore, Amsterdam, The Netherlands]), respectively, for 1 h.

420 The gels were washed for 5 min in blotting buffer (14.4 g/l glycine, 3.03 g/l, Tris-HCl, 20%

421 ethanol) and Whatman Protran nitrocellulose membranes (BA85, pore size 0.45 µm [Sigma-

422 Aldrich]) were activated in blotting buffer for 5 min as well. Proteins were transferred onto

423 nitrocellulose membranes with the TransBlot Turbo (BIORAD, Hercules, California) for 30

424 min at 25 V and 1 A in 190 mM glycin, 25 mM Tris, 15% methanol or onto Immobilon-FL

425 membranes (Merck-Millipore) at 125 V for 55 min in blotting buffer. The membranes were

426 blocked for 1 h using Odyssey Blocking Buffer (LI-COR, Hamburg, Germany) diluted 1:1 with

427 Tris-buffered saline (TBS; 50 mM Tris-HCl and 150 mM NaCl, pH 7.4). PK, HMWK and FXII

428 were detected with affinity-purified polyclonal antibodies at 1:4000 (CL20090A, CL20027AP

429 and CL20055AP, respectively, Cedarlane, Burlington, Canada) by overnight incubation at

430 4°C. Membranes were washed with TBS, 0.05% Tween-20 and probed with Donkey-anti-

431 goat-IgG DyLight800 conjugate (C30530-01, LI-COR) at 1:40000 or Alexa Fluor 680 donkey

432 anti-sheep IgG 1:5000 (A-21102, Thermo Fisher Scientific, Waltham, Massachusetts) for 1 h

433 at room temperature. Membranes were washes again with TBS, 0.05% Tween-20 and

434 images were developed using the Fusion FX (Vilber Lourmat, Eberhardzell, Germany) or a

435 near-infrared Odyssey scanner (LI-COR) with excitation/emission at 640 nm/680 nm or 740

436 nm/800 nm.

437

438 *Mass Spectrometry*

439 FXII proteins (WT and W268R, full-length and cleavage products) planned to be analyzed by

440 mass spectrometry were applied to 10% SDS-PAGE as described above (1 µg/lane), stained

441 over night at 4°C using Instant Blue (ISB1L, Expedeon San Diego, California) and destained

442 for ≥1h at ambient with destaining solution (10% acetic acid, 20% ethanol). Defined bands

443 were cut by scalpel and sent for mass spectrometry at BIH Proteomics (Max Delbrück

444 Center, D-13125 Berlin). Tryptic digest was performed in-gel on defined bands (Fig. 2B I, II,

445 III, IV). Each band was cut into small pieces, washed with 50 mM ABC (ammonium

446 bicarbonate) and 50% ethanol/50 mM ABC buffer, dehydrated with 100% Ethanol and

447 vacuum dried, incubated for 45 min with 10 mM DTT at 56°C, followed by 45 min incubation

448 with 55 mM chloroacetamide (Merck-Millipore). After one round of 50 mM ABC and 50 %

449 ethanol/50 mM ABC buffer wash, gel pieces were dehydrated with 100% ethanol, dried and

450 each sample was incubated with 1 µg trypsin (sequence-grade modified, V5111, Promega,

451 Madison, Wisconsin) in 50 mM ABC buffer over night at 37°C. After acidifying the samples

452 with formic acid (2% final concentration), peptides were extracted with 30% ethanol/50 mM

453 ABC followed by 100% acetonitrile. The supernatants were combined and dried down. The

454 resulting peptide pellet was dissolved in 3% acetonitrile/0.1% formic acid and analyzed by

455 mass spectrometry. For mass spectrometric measurements, the peptide mixture was

456 separated by reversed-phase chromatography using an EasyLC 1200 system (Thermo

457 Fisher Scientific) on in-house-manufactured 20 cm fritless silica microcolumns packed with

458 ReproSil C18 resin (inner diameter 75 µm, Dr. Maisch GmbH, Ammerbuch-Entringen,

459 Germany). Peptides were separated using an 8-60% acetonitrile gradient (44 min length) at a

460 nanoflow rate of 250 nl/min. Eluting peptides were directly ionized by electrospray ionization

461 and analyzed on a Q Exactive HF-X (Thermo Fisher Scientific). Mass spectrometry was

462 performed in data-dependent positive mode with one full scan (m/z range = 350 - 1800; R =

463 60000; target value: 3×10^6; maximum injection time = 45 ms). The Top 20 most intense ions

464 with a charge state between 2 and 7 were selected (R = 45000, target value = 1×10^5;

465 isolation window = 1.3 m/z; maximum injection time = 86 ms). Dynamic exclusion for

466 selected precursor ions was set to 20 s. Data analysis was performed using MaxQuant

467 software package (version 1.6.0.1[46]). The internal Andromeda search engine was used to

468 search MS^2 spectra against human coagulation factor XII wild type and W268R mutant

469 sequence containing forward and reverse sequences. A database for contaminants was

470 included. The search included variable modifications of methionine oxidation, N-terminal

471 acetylation, deamidation (N and Q) and the fixed modification of carbamidomethyl cysteine.

472 Minimal peptide length was set to six amino acids, digestion parameter was semitryptic. The

473 FDR was set to 0.01 for peptide, protein and site identifications. To filter for confidently

474 identified peptides, a minimum of 2 MS counts and valid value in both replicates for at least

475 one sample group was required. Intensity values were log2 transformed. Mass spectrometry

476 was performed twice; n=2 duplicates were assessed for each band.

477

478 *Factor XII protein expression and purification*

479 The eukaryotic expression vector pcDNA6/V5-His A (Thermo Fisher Scientific) was modified

480 by insertion of a murine Igκ secretion signal and two strep-tags through the HindIII and EcoRI

481 digestion sites (called pSM2). cDNA of the F12 gene was kindly donated by Dr F. Citarella

482 and matches the National Center for Biotechnology Information reference sequence

483 (NM_000505.3), except for the silent mutation c.nt.1140T>C (P460P, mature protein

484 numbering). A sequence coding for a tobacco etch virus (TEV) protease cleavage site was

485 inserted from the F12 sequence by using PCR with the Phusion Polymerase (Thermo Fisher

486 Scientific). FXII-W268R was developed through two-step assembly of three double-stranded

487 DNA segments (annotated as N, M and C, respectively. Primer sequences in Table 2).

488 Fragment N with the desired mutation (T859A), was ordered as double stranded DNA

489 fragments from Integrated DNA technologies (Coralville, Iowa) and ligated into the

490 pJET1.2/blunt vector (Thermo Fisher Scientific) according to manufacturer instructions and

491 amplified through PCR. Fragments M and C, were amplified by PCR from FXII-WT cDNA in

492 pSM2. First, the N and M segments were digested with XmaI and subsequently, fused by

493 ligation with T4 ligase (Thermo Fisher Scientific). The fused product (NM) was amplified via

494 PCR. Hereafter, the NM and C segments were digested with BsaI and fused together by

495 ligation with T4 ligase. The assembled cDNA product was ligated into the pJET1.2/blunt

496 vector according to manufacturer's instructions. Inserts were digested with EcoRI and NotI

497   and ligated into the EcoRI and NotI-restricted pSM2 vector. All restrictases were purchased

498   from New England Biolabs (Ipswich, Massachusetts). Sequences were verified by Sanger

499   sequencing (Macrogen Europe, Amsterdam, The Netherlands) prior to transfection.

500   HEK293F (LGC Standards GmbH, Wesel, Germany) cells were transfected using 293fectin

501   (Thermo Fisher Scientific) and cultured in Gibco FreeStyle 293 Expression Medium (Thermo

502   Fisher Scientific) at a concentration of 1 x 10^6 cells/ml in 2 ml. Four days after transfection,

503   the cell suspension was centrifuged and 500 x g and the supernatant was collected and

504   stored until use at -20°C. For protein purification, transfection was repeated in a volume of 1 l

505   cell suspension and culture medium was harvested after 1 week. FXII-W268R was purified

506   via a strep-tag using Strep-Tactin Sepharose beads (IBA-Life Sciences, Goettingen,

507   Germany) and dialyzed over night at 4°C against 4 mM sodium acetate–HCl with 150 mM

508   NaCl (pH 5.5) using a Spectra/Por dialysis membrane (molecular weight cut-off 3.5 kDa,

509   Spectrum Labs, Rancho Dominguez, California) that was preincubated with polybrene

510   (Sigma-Aldrich).

511   *Chromogenic assays*

512   Chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mM, Bachem, Bubendorf, Switzerland)

513   was used to detect FXII activity from recombinant constructs in culture medium. 96-well

514   Costar plates (Corning, New York) were blocked with HBS (10 mM HEPES [VWR

515   International, Amsterdam, The Netherlands], 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl,

516   pH=7.4) containing 0.1% BSA and subsequently, 40 µl culture medium supernatant

517   containing rFXII was added followed by 10 µl substrate. Substrate conversion was

518   immediately read out at 405 nm at 37°C. FXII activity in plasma was assessed using the

519   Chromogenic Assay for Factor XIIa-like Activity (COA0088, CoaChrom Diagnostica, Maria

520   Enzersdorf, Austria) as specified by the manufacturer. Absorbance signals were measured in

521   the VIKTOR X5 (PerkinElmer, Waltham, Massachusetts) and analyzed using GraphPad

522   Prism 7 or Microsoft Excel 2016.

523

Factor XII concentration ELISA

FXII concentration in plasma was assessed using the Matched-Pair Antibody Set ELISA of human Factor XII antigen (FXII-EIA, CoaChrom Diagnostica) as specified by the manufacturer. Absorbance signals were measured in the VIKTOR X5 (PerkinElmer, Waltham, Massachusetts) and analyzed using GraphPad Prism 7 or Microsoft Excel 2016.

PK activity (routine lab) and PK ELISA

Prekallikrein activity was assessed by coagulometric assay in routine measurements at Labor Berlin (13353 Berlin, Germany). Prekallikrein plasma concentration was measured using the Prekallikrein and Kallikrein Human ELISA (ab171015, Abcam, Cambridge, UK) as specified by the manufacturer. Absorbance signals were measured in the VIKTOR X5 (PerkinElmer, Waltham, Massachusetts) and analyzed using GraphPad Prism 7.02 or Microsoft Excel 2016.

Enzyme-C1-INH complexes ELISA

Methods on V_HH (variable domain heavy-chain-only antibody) selection, production and execution for this assay are described earlier by de Maat 2016.[25] In brief, V_HH 1B12 (5 µg/ml), a monoclonal V_HH directed against complexed C1-INH, was immobilized on 96-well Maxisorp plates (Corning). Plasma samples were diluted in 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH=7.4 (HBS) supplemented with 0.1% (w/v) Tween-20 and 1% (w/v) skimmed milk powder (Sigma-Aldrich) and containing 200 µM Phe-Pro-Arg-chloromethylketone (PPACK, Haematologic Technologies). For reference, normal pooled plasma from healthy donors (NPP) activated with DXS (average molecular weight 500.000, Sigma-Aldrich) for 30 min as well as non-activated NPP were prepared in HBS containing PPACK and mixed to generate samples containing 0%-100% DXS activated sample. Samples were incubated on the plate for 1h. Enzyme present in the captured complexes was

550   detected with a biotinylated polyclonal V_HH targeted against the βFXIIA (5 µg/ml, made and

551   biotinylated in-house[47]) followed by streptavidin-poly-HRP 1:4000 (M2051, Sanquin Blood

552   Supply, Amsterdam, The Netherlands) or a polyclonal sheep anti-PK IgG 1:2000 (SAPK-AP,

553   Affinity Biologicals, Hamilton, Canada) followed by HRP-labeled rabbit anti-sheep IgG 1:4000

554   (P0163, Dako, Heverlee, Belgium). Finally, 3,3′,5,5′-tetramethyl-benzidine (TMB, Tebu Bio,

555   Heerhugowaard, The Netherlands) was added and substrate conversion was stopped after

556   5 to 10 min with H_2SO_4 (0.3 M) and absorbance was read out at 450nm. GraphPad Prism

557   7.02 software was used to interpolate data from the reference range.

558

559   *Cleaved kininogen activity ELISA*

560   Methods on V_HH selection, production and execution for this assay are described more

561   detailed by Hofman and colleagues.[48] In brief, patients samples were diluted 64-fold in DPBS

562   (Thermo Fisher Scientific) supplemented with 0.1% (w/v) Tween-20 and 1% (w/v) skimmed

563   milk powder and containing 200 µM PPACK and 0.5 µg/ml DXS to generate HMWK

564   complexes. For reference, 5 min βFXIIa activated NPP and non-activated NPP samples were

565   prepared and mixed to generate samples with various amounts of cHMWK. After incubation,

566   cHMWK was detected using a biotinylated monoclonal anti-HMWK V_HH H4 (2 µg/ml, made

567   and biotinylated in-house) and streptavidin-poly-HRP 1:2000. TMB substrate conversion was

568   measured and analyzed as described for the enzyme-C1INH complex assays.

569

570   *Bradykinin ELISA*

571   Bradykinin levels in patient and healthy control plasma were assessed by ELISA (ADI-900-

572   206, Enzo Life Sciences, Farmingdale, New York) as described by the manufacturer.

573   Absorption signals were measured in the VIKTOR X5 and analyzed using GraphPad Prism

574   7.02 or Microsoft Excel 2016 against bradykinin standard curves.

575

576 *IL-1β and IL-8 ELISA*

577 IL-1β/IL-8 secretion was assessed by ELISA (DuoSet, DY201/DY208-05, R&D Systems,

578 Minneapolis, Minnesota) as specified by the manufacturer. For greater precision, ELISAs

579 were carried out at ¼ of the recommended volumes per well in black NUNC 384-well

580 MaxiSorp plates (Thermo Fisher Scientific) for 4 x replicate measurements per original 96-

581 well cell culture plate well. Additionally instead of TMB, we developed the assay using

582 luminol/H_2O_2 for increased sensitivity (Amersham ECL Prime, GE Healthcare, Chicago,

583 Illinois). Luminescence signals were measured in the VIKTOR X5 and analyzed using

584 GraphPad Prism 7.02 or Microsoft Excel 2016 against IL-1β/IL-8 standard curves.

585

586 *Immunofluorescence Cytology / Histology*

587 Neutrophils from patient and healthy donor blood were isolated using the MACSxpress

588 Neutrophil isolation Kit (130-104-434, Miltenyi Biotec, Bergisch Gladbach, Germany) as

589 specified by the manufacturer. Isolated neutrophils were washed once with DPBS and ca.

590 10^5 cells were applied to cytospin. Briefly, cells were concentrated to dense spots by

591 centrifugation onto SuperFrost Plus microscopy slides (Thermo Fisher Scientific) through

592 perforated cytospin microtubes (Frötek-Kunststofftechnik GmbH, Osterode am Harz,

593 Germany) and Heraeus perforated cytospin filter cards (Thermo Fisher Scientific) with

594 subsequent fixation by acetone at -20°C. Cytospins were encircled by PAP pen, blocked with

595 Serum-free protein block (S3022, Dako) for 10 min at RT and incubated with primary

596 antibody against FXII 1:100 (sc-376770, Santa Cruz Biotechnology, Dallas, Texas) or IL-1β

597 1:100 (LS-C174781, LSBio, Seattle, Washington) in 2% goat normal serum in TBS for 3 h at

598 RT. After rinsing 3 x 5 min with TBS, goat anti-mouse IgG Alexa Fluor 594 conjugate (115-

599 585-062, Jackson Immuno Research, West Grove, Pennsylvania) was applied 1:200 in 2%

600 goat normal serum in TBS for 30 min in the dark, again with subsequent 3 x 5 min rinsing

601 with TBS. In case of double stainings, primary antibody against MPO (MAB3174, R&D

602 Systems) was incubated together with goat anti-mouse IgG Alexa Fluor 488 conjugate (115-

22

603   545-146, Jackson Immuno Research) 1:100 and 1:200, respectively, in 2% goat normal

604   serum in TBS for 1 h in a microtube in the dark. Free anti-mouse IgG was blocked with 4%

605   final normal mouse serum for 1 h in the dark and antibody-antibody-dye conjugate was

606   applied to the cytospins and incubated overnight at 4°C in the dark. After rinsing 3 x 5 min

607   with TBS, slides were air-dried briefly, mounted using DAPI-Fluoromount G (Thermo Fisher

608   Scientific) and air-dried in the dark for several hours before microscopy. Patient biopsy was

609   fixed in 4% buffered formalin overnight at RT and paraffinated using an automated

610   histokinette (Shandon Citadel 2000 Tissue Processor, Thermo Fisher Scientific). 5 μm skin

611   tissue sections were applied to SuperFrost Plus slides, baked at 60°C for 1 h and

612   subsequently deparaffinated with 2 x 10 min xylene, 2 x 2 min 100% ethanol, 2 x 2 min 96%

613   ethanol, 2 min 70% ethanol and 3 min ddH$_2$O. Antigens were retrieved by incubation at 95°C

614   in citrate buffer (1.8 mM citric acid, 8.6 mM Na$_3$ citrate, pH=6) in a humid atmosphere for 15

615   min. Tissue sections were rinsed in TBS, encircled with PAP pen and stained as described

616   above. Samples of liver tissue (kindly provided by Charité Dpt. of Pathology) served as

617   positive control for FXII. Sections omitting the primary antibody served as negative controls.

618   Slides were analyzed by fluorescence microscopy using a Zeiss Axioplan 2 with Axiovision

619   software 4.8.1. Images of cytospins were further analyzed by ImageJ 1.52a.

620

621   *Immunohistochemistry*

622   Paraffin sections (5 μm) of a FACAS patient skin sample and control skin were prepared as

623   explained above and processed for routine (hematoxylin/eosin) and immunohistological

624   stainings. We used an immunohistochemistry protocol (streptavidin-biotin labelling)

625   employing primary antibodies targeting MPO (MAB3174, R&D Systems) 1:400 overnight at

626   4°C or CD163 (ab189915, Abcam) 1:400 overnight at 4°C. After washing 3 x 5 min with TBS,

627   REAL Detection System Alkaline Phosphatase/RED (K5005, Dako) for MPO staining or

628   Labelled polymer-HRP anti-rabbit (K4011, Dako) together with AEC+ High Sensitivity

629   Substrate Chromogen Ready-to-use (K3461, Dako) for CD163 staining were applied

630 according to the manufacturer's instructions and subsequently analyzed by brightfield

631 microscopy. Samples of tonsil tissue served as positive controls. Sections omitting the

632 primary antibody served as negative controls.

633

634 *PBMC stimulation assays*

635 Venous blood from patients and healthy donors was sampled into heparin tubes. 15 ml of

636 Ficoll-Paque solution (GE Healthcare) were pipetted into 50 ml Leucosep tubes (Greiner Bio

637 One) and centrifuged at 1000 x g for 1 min. Up to 20 ml of blood sample per tube were

638 added, filled ad 50 ml with DPBS and centrifuged for 10 min at 1000 x g with 33%

639 acceleration and without breaks. The upper plasma layer was discarded, the PBMC layer

640 was collected and filled into a new 50 ml tube. PBMCs were washed twice by adding ad 50

641 ml DPBS, centrifugation for 10 min at 350 x g and resuspension in residual DPBS. After the

642 washing steps, 10 ml of PBMC medium (RPMI 1640 containing 1% FBS [both from

643 Biochrom, Berlin, Germany], 100 U/ml penicillin, 100 µg/ml streptomycin [HyClone 100x

644 Pen/Strep, GE Healthcare]) was added and cells were counted using trypan blue staining

645 and hematocytometer. PBMC number was adjusted to 3 x 10^6/ml and 100 µl of the

646 suspension were added per well to Falcon TC treated 96 well plates (Corning). After 2 h

647 incubation at 37°C and 5% CO_2, the supernatant containing non-monocytes was discarded

648 and to the adherent monocytes, 100 µl of containing 0.5 ng/ml LPS (*E.coli* R515, Enzo Life

649 Sciences) or not was added for NF-κB priming. Monocytes were incubated for 20 h at 37°C

650 and 5% CO_2, supernatant was discarded and 20 µl per well of PBMC medium was added. 20

651 µl of either bradykinin at stated final concentrations or FXII constructs (with or without stated

652 final concentrations of PPACK) diluted in PBMC medium were added at a final concentration

653 of 35 ng/µl, followed by incubation at 37°C and 5% CO_2 for 1 h. Subsequently, 10 µl of 10

654 mM ATP (Sigma Aldrich) diluted in PBMC medium were added to a final concentration of 2

655 mM for inflammasome activation, followed by another 1 h incubation at 37°C and 5% CO_2.

656 Supernatants were collected and stored at -20°C until analysis by ELISA.

24

657

Statistical analysis

659 Sample sizes were limited due to patient availability. The number of samples (all assessed in

660 duplicates) and experiments are displayed and listed in the figure legends. The use of

661 statistical tests is indicated in the figure legends as well.

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Data availability

669 Source data for figure numbers 2a,b,c,d,e, 3a,c,d,e,f,g,h, 4b,c, 5a,b,c and supplementary

670 figures 1, 2 and 4 are provided as a Source Data file

671 (https://doi.org/10.6084/m9.figshare.11117669).[49] The mass spectrometry proteomics data

672 have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner

673 repository with the dataset identifier PXD016341. As the informed consent obtained from

674 FACAS patients does not allow for public deposition of the sequencing data, the filtered WES

675 sequencing data from patients #2, #5 and #7 can be communicated upon reasonable request

676 to R.J.P. and S.E. All other relevant data are contained within the article and its

677 supplementary   information.

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836 **Author contributions**

837 JS: Supervision of experiments, critical input on study concept and manuscript

838 NM: Performed cell stimulation assays on IL-1ß response of PBMCs, Western blot, ELISA,

839 graphs and stats

840 ZH: Western blot, ELISA and substrate assay data on contact system activation in patient

841 plasma and activity of recombinant FXII, graphs and stats

842 JW: Performed cell stimulation assays on IL-1ß response of PBMCs, ELISA

843 HB: Performed and analyzed immunohistochemistry

844 SdM: Developed synthetic genetic FXII constructs, Western blot analysis

845 RJP: Performed exome analysis

846 SE: Concept for genetic analysis and analysis of sequencing data

847 JH: Critical input on concept for genetic analysis

848 MK: Performed and analyzed mass spectrometry data

849 PM: Supervised and discussed mass spectrometry analysis

850 MKC: Critical input on manuscript

851 MMau: Critical input on study concept and manuscript

852 CM: Supervision of experiments on contact system, critical input on study concept

853 KK: Overall study concept and idea; supervision of experiments and collection of clinical

854 data; data analysis; wrote the manuscript with contributions from all authors

855 All authors critically reviewed the manuscript and approved the final version before

856 submission.

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859 **Competing interests**

860 The authors declare the following competing interests:

861 MMau received consultation fees and research support from Shire/Takeda outside the

862 submitted work. KK received research support from Shire/Takeda outside the submitted

863 work. The other authors declare no conflict of interest.

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880 **Figure legends**

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882 **Fig. 1: Phenotype and genotype in patients with FXII-associated cold**
883 **autoinflammatory syndrome (FACAS). a)** Urticarial rash of the face of a 9-year-old girl and
884 the leg of her 35-year-old mother. **b)** Skin histopathology reveals dermal edema (H.E. yellow
885 arrow) and perivascular infiltrates composed of macrophages (CD163) and few intravascular
886 neutrophils (myeloperoxidase [MPO], yellow arrow). Original magnification 400x (n=2
887 technical replicates). **c)** Family pedigree over four generations identifying affected (shaded
888 black) and unaffected (unshaded) individuals. Exome sequenced individuals are underlined.
889 Where conducted, results of Sanger sequencing of p.W286R are shown to top left of each
890 individual whereby '+' denotes heterozygote for variant and wild type and '-' denotes healthy
891 reference. **d)** Representative sequencing chromatogram of the heterozygous genotype in an
892 affected individual (top) and chromatogram of healthy individual from the same region
893 (bottom).

894

895 **Fig. 2: FXII W268R results in fragmentation and spontaneous activation of FXII. a)** FXII
896 fragmentation in FACAS plasma samples and FXII W268R mutant proteins. Citrate plasma
897 of affected (#1, #2, #5) and non-affected (#3, #4, #6) family members, recombinant wild type
898 and W268R FXII expressed in HEK293 cells and plasma from an FXII-HAE patient was
899 separated by SDS-PAGE (reducing blot) and immunoblotted for FXII. Red arrows at 50 kDa
900 indicate heavy chain of cleaved FXII in the plasma of affected patients and recombinant

31

901 W268R FXII (rFXII W268R), which is barely visible in non-affected and hereditary

902 angioedema (HAE) III plasma or wild-type FXII (rFXII wt). Recombinant W268R and wild-

903 type FXII were expressed in HEK293 cells. Black arrows point to rFXII W268R fragments

904 observed by mass spectrometry in 2c, n=5 technical replicates. **b)** Immunoblot (reduced

905 conditions) of recombinant mutant proteins (in the presence or absence of surface activator

906 kaolin) and controls (rFXII wt and purified αFXIIa). In addition to 50 kDa (heavy chain, HC)

907 and 25 kDa (light chain, LC) bands, a further band appears at 60 kDa (II) that is absent in

908 rFXII wt and αFXIIa. Black arrows point to rFXII W268R fragments observed by mass

909 spectrometry in 2c, n=3 technical replicates. **c)** Schematic presentation of FXII W268R

910 procession. Red arrows point to mutation at position W268R. Proteolytic sites are indicated

911 by blue arrows. Cleavage after position 353 results in FXIIa W268R HC (50 kDa) and FXIIa

912 W268R LC (25 kDa) fragments. Additional cleavage results in a truncated FXII W268R

913 fragment (60 kDa) cleaved at position 447. **d)** FXIIa activity was measured by a chromogenic

914 assay demonstrating considerable spontaneous FXIIa activity in plasma of affected

915 individuals (n=3) but less in healthy donors (n=3 healthy family members). **e)** Spontaneous

916 FXIIa activity was also observed in culture supernatants from HEK293 cells expressing

917 W268R but not wild-type FXII. Graphs show individual data points and the mean ±sem.

918 Statistical significance is indicated by unpaired Student's t-test with Welch's correction (d,e).

919 Source data are provided as a source data file.

920

921 **Fig. 3: FXII W268R activates the kallikrein-kinin pathway. a)** Citrate plasma of affected

922 (n=3) and healthy family members (n=3) was immunoblotted for prekallikrein (PK) showing

923 substantial cleavage of PK (black arrow) to kallikrein (red arrow), n=2 technical replicates.

924 This corresponds to reduced PK activity **(b)** and plasma levels **(c)** in affected individuals (n=3

925 affected vs. n=3 healthy). **d)** FXIIa-C1-inhibitor (FXIIa-C1-INH) and **e)** PK-C1-INH complexes

926 in patient plasma (n=3 affected vs. n=3 healthy). **f)** Citrate plasma of affected (n=3) and

927 healthy family members (n=3) was immunoblotted for high molecular weight kininogen

928 (HMWK) showing its complete degradation (black arrow) to cleaved HMWK (cHMWK) (red

929 arrow), n=2 technical replicates. Correspondingly, increased cHMWK activity **(g)** and

930 bradykinin plasma levels **(h)** (n=3 affected vs. n=3 healthy; n=2 affected vs. n=7 healthy and

931 n=2 HAE I/II) are detectable in the plasma of affected patients. PK and cHMWK activity were

932 measured by chromogenic assays, PK and bradykinin plasma levels were assessed by

933 ELISA. Graphs show individual data points and the mean ±sem. Statistical significance is

934 indicated by unpaired Student's t-test with Welch's correction (b,c,d,e,g) or 1-way ANOVA

935 with Sidak's multiple comparisons test (h). Source data are provided as a source data file.

936 **Fig. 4: FXII is expressed by skin and blood neutrophils in FACAS. a)** Representative

937 image from serial sections of lesional skin of FACAS patient ca. 1h after cold exposure. FXII

938 immunoreactivity is restricted to neutrophils at perivascular sites and within blood vessels

939 (white arrows), n=2 technical replicates. **b)** Exemplary images from cytospins isolated from

940 the peripheral blood of FACAS patients and healthy control subjects. Only single FACAS

941 neutrophils (0.72%), but not healthy control neutrophils (0%) present with double-positive

942 cytoplasmatic staining for FXII and MPO. Original magnification 400x, n=3 technical

943 replicates. **c)** Peripheral blood mononuclear cells were isolated from healthy control subjects

944 by density gradient centrifugation and stimulated with increasing doses of bradykinin. IL-8

945 secretion was assessed by ELISA. Pooled data of n=3 different donors, n=2 technical

946 replicates. The graph shows individual data points and the mean ±sem. Statistical

947 significance is indicated by 1-way ANOVA with Sidak's multiple comparisons. Source data

948 are provided as a source data file.

949

950 **Fig. 5: FXII W268R induces and increases IL-1ß secretion and expression. a-c)**

951 Peripheral blood mononuclear cells were isolated from healthy control subjects by density

952 gradient centrifugation. Interleukin-1ß (IL-1ß) secretion requires a two-step process: i)

953 transcriptional upregulation of pro-IL-1ß via toll-like receptors and ii) conversion of pro-IL-1ß

954 into its active form by inflammasome activation. Cells were pre-stimulated with toll-like

955 receptor agonist lipopolysaccharide (LPS) and nucleotide binding like receptor protein 3

956 (NLRP3) inflammasome activation was induced by the danger molecule ATP (LPS + ATP =

957 blue symbols). Red symbols denote LPS-stimulated cells only and green symbols represent

958 ATP-activated cells without priming by LPS. Experiments were performed in the presence of

959 FXII-depleted plasma (control) and addition of recombinant FXII wild-type (rFXII wt) and

960 W268R (rFXII W268R) protein **(a)** or rFXII W268R-S544A **(b)**. Experiments in **(c)** were

961 conducted with purified FXII, FXIIa and different doses of the small peptide-based inhibitor

962 PPACK. Black arrows indicate priming effect by the mutant rFXII W268R **(a)** and the active

963 site incapacitated rFXII W268R-S544A **(b)** which was absent in rFXII wt and control. Also,

964 the presence of FXIIa (with or without PPACK) did not induce changes in IL-1ß release **(c)**.

965 IL-1ß secretion was assessed by ELISA. Pooled data of n=3 different (a) or n=2 (b,c)

966 experiments. Bar graphs show individual data points and the mean ±sem. Statistical

967 significance in is indicated by 1-way ANOVA (a,c) with Tukey's multiple comparisons test for

968 ATP between the groups control vs. rFXII W268R and rFXII wt vs. rFXII W268R (a) or

969 unpaired Student's t-test (b). **d,e)** Representative image from serial sections of lesional skin

970 from FACAS patient following cold exposure exhibits IL-1ß immunoreactivity. IL-1ß

971 expression is pronounced around blood vessels (yellow arrows) and co-localizes with

972 neutrophils (**d**) and macrophages (**e**) (white arrows). Original magnification 400x, n=2

973 technical replicates. Source data are provided as a source data file.

974

975 **Fig. 6: The bradykinin B2 receptor antagonist icatibant resolves FACAS signs and**

976 **symptoms.** During winter with continuous skin symptoms, the index patient administered

977 icatibant 30mg. **a)** Urticarial rash completely resolved within 90 min post icatibant application

978 (from left to right). **b)** Total and individual symptoms reported and assessed daily over a time

979 period of 8 days by the patient using a standardized daily health assessment form covering

980 the main symptoms rash, fatigue, headache, arthralgia and fever/chills. The total score

981 ranges from 0 = no symptoms to 50 = maximum of symptoms. Each subscore can range

982 from 0 = no symptoms to 10 = maximum of symptoms. The subscores do not show

983 fever/chills as these symptoms were not present at that time. Icatibant was given on four

984 consecutive days (red arrows).

985

986 **Fig. 7: Proposed pathomechanism underlying FXII W268R mutation in FACAS.** Liver-

987 derived and pre-activated FXII induces plasma prekallikrein cleavage, HMWK cleavage

988 leading to constantly raised bradykinin (BK) levels in the patient plasma (1) and increased

989 permeability of the vasculature (3). BK may also induce IL-1ß release from monocytes and

990 tissue macrophages (Mϕ) leading to the autoinflammatory phenotype observed in FACAS

991 patients (2), (5). IL-1ß may further induce BK accumulation by upregulation of its receptors

992 and downregulation of its inactivating enzyme ACE (4). In the skin, BK activates Mϕ to

993 produce IL-8 and IL-1ß, recruiting neutrophils into the tissue (5). These neutrophils may act

994 as a source of auto-activated FXIIa in the skin, which leads to further contact system

995 activation and BK generation (6). Under normal conditions C1-Inhibitor can control BK

996 generation, however, upon cold exposure inhibitory capacity may drop leading to a local

997 increase in BK. Activation of neutrophils may occur via a paracrine feedback loop involving

998 plasma kallikrein and HMWK. In addition, BK may activate skin mast cells to release heparin

999 and polyphosphates (7), which supports further activation of FXII.

1000

1001 **Table 1:**

1002 **Individual clinical characteristics and manifestations in FACAS patients**

Characteristic	Patient ID #1	Patient ID #2	Patient ID #5	Patient ID #7
Age at diagnosis	35	64	9	27
Sex	Female	Male	Female	Female
Age at disease onset	After birth	After birth	After birth	After birth
Skin symptoms	Cold-induced urticarial rash	Cold-induced urticarial rash	Cold-induced urticarial rash	Cold-induced urticarial rash
CNS symptoms	Headache	Headache	Headache	Headache
Musculo-skeletal symptoms	Moderate arthralgia	Severe arthralgia	None	Mild arthralgia
Systemic symptoms	Fatigue, subfebrile temperatures	Fatigue, subfebrile temperatures	Fatigue	Fatigue
S100 A12 in ng/ml (Ref. <200)	523.7		1024.3	-
S100 A8/9 in µg/ml (Ref. <2.94)	-	4.10	-	4.51
Serum amyloid A in mg/l (Ref. <6.40)	16.80	n.a.	n.a.	8.20

1003

1004 **Table 2:**

1005 **Primer sequences for FXII genetic constructs**

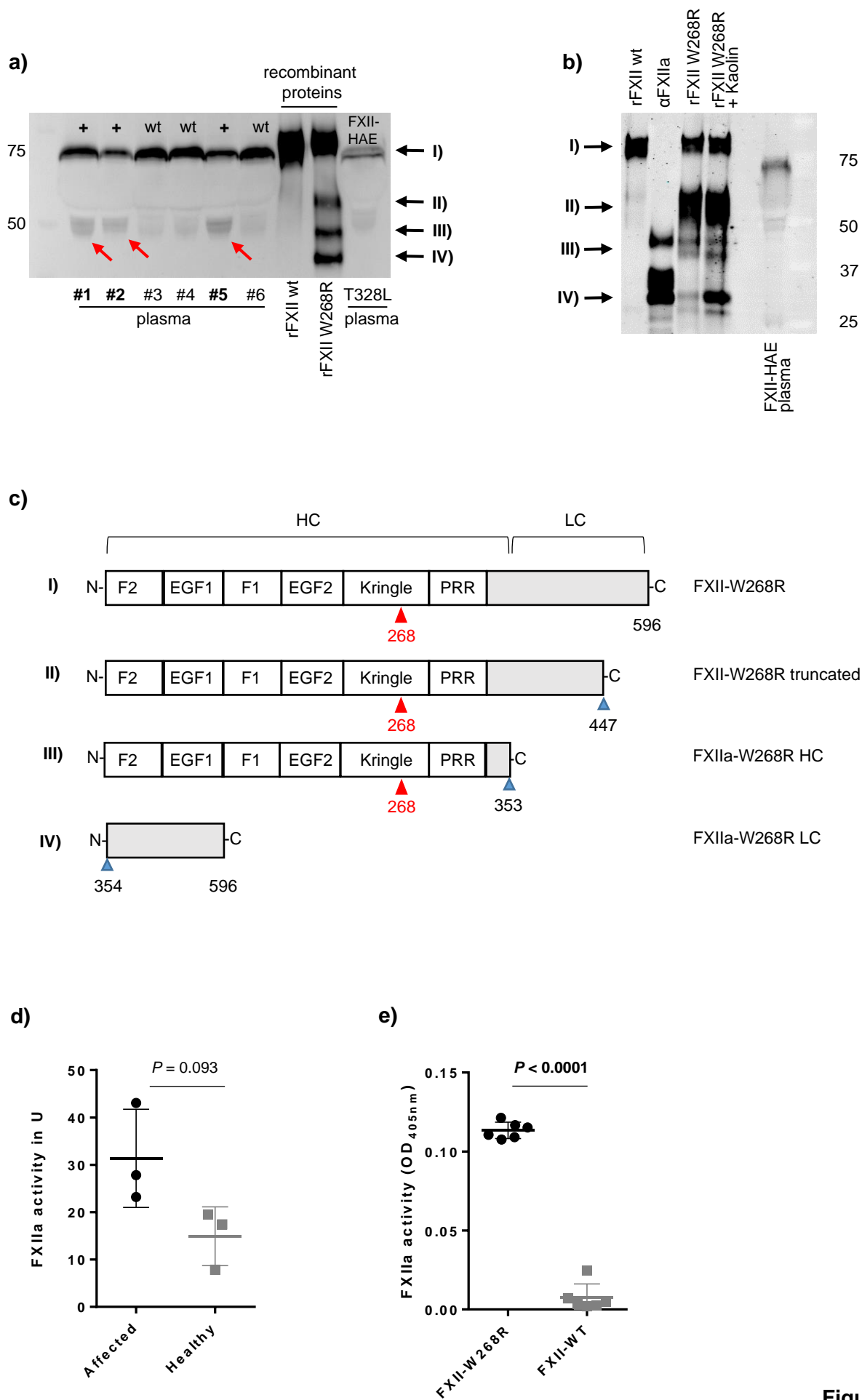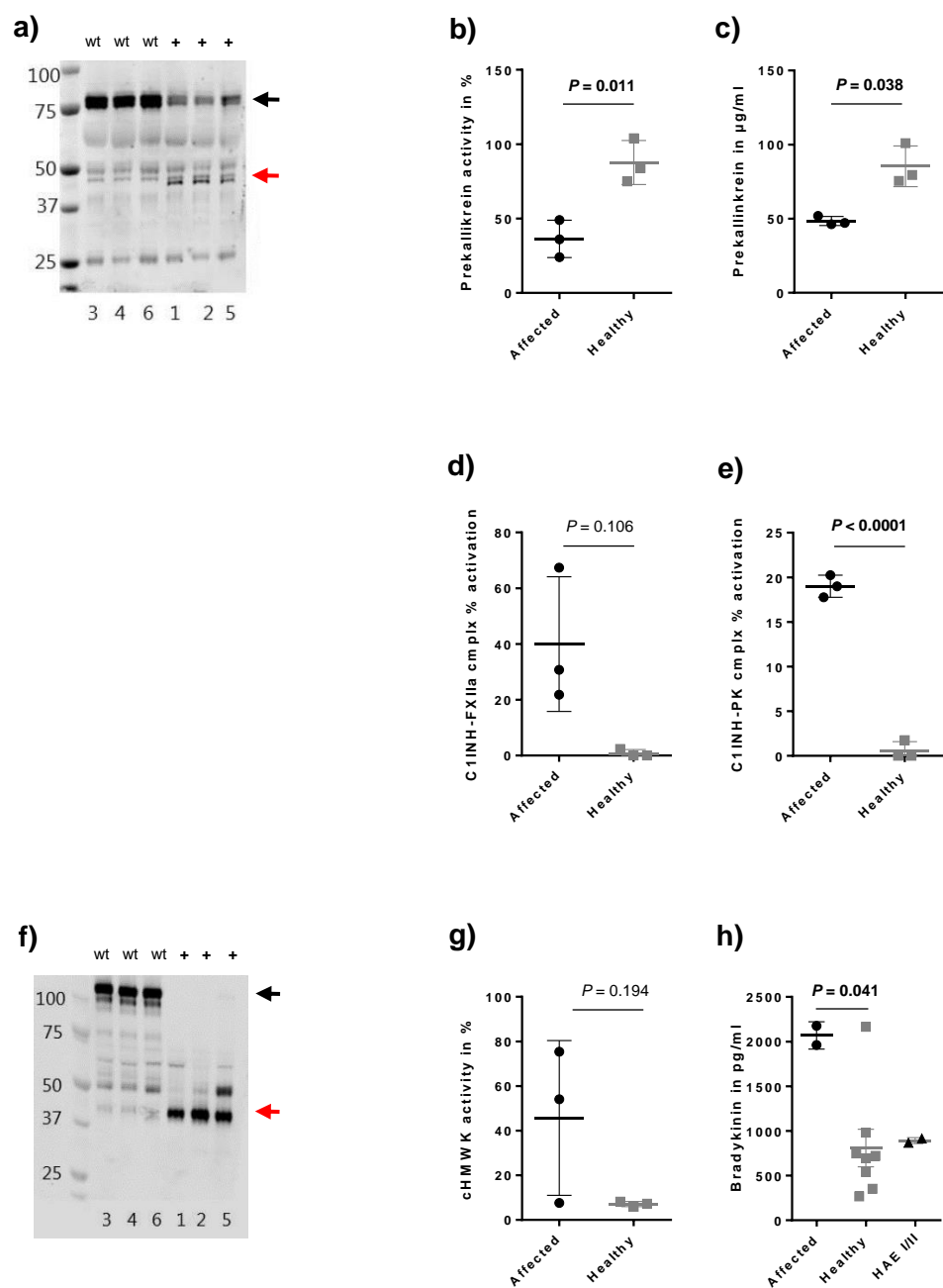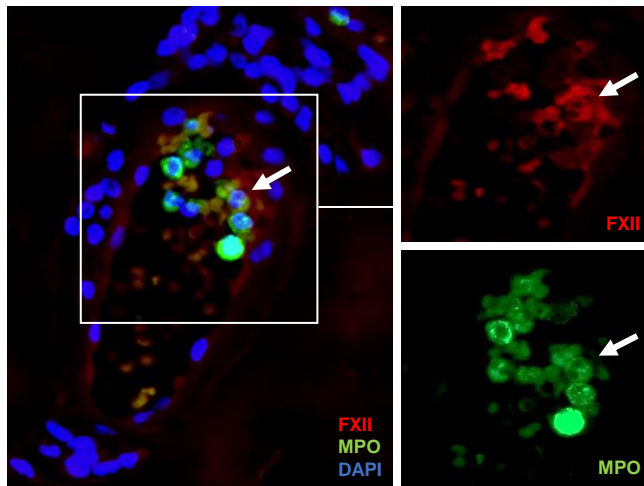|	
N-Frag_For	GGAGAATTCGAAAACCTGTATTTTCAGTCTATTCCACC
N-Frag_Rev	GCTCCCGGGGTCTGGGACTGAGGCGGGG
M-Frag_For	GACCCCGGGAGCCTTGCCGGCGAAGCG
M-Frag_Rev	CGTGGTCTCGGAGGGTCGCGCGGCGCCGCTTG
C-Frag_For	GACCCTCCGAGACCACGCTCTGCCAGG
C-Frag_Rev	CGAGCGGCCGCTCATCAGGAAACGGTGTGCTCCCG

1006

1007

a)

b)
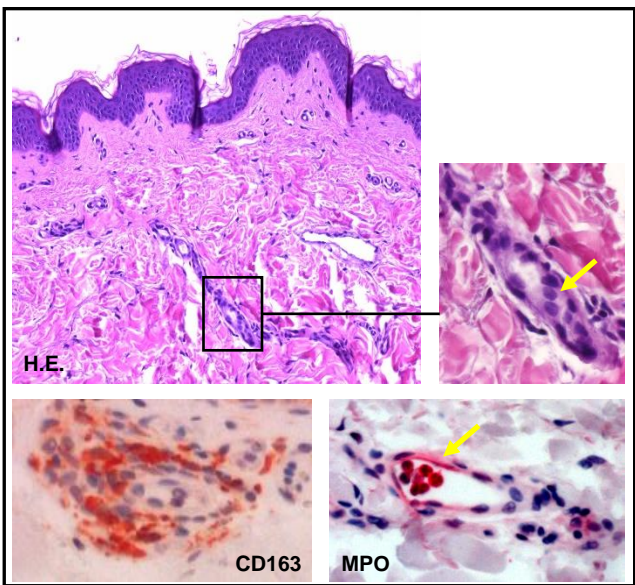
c)
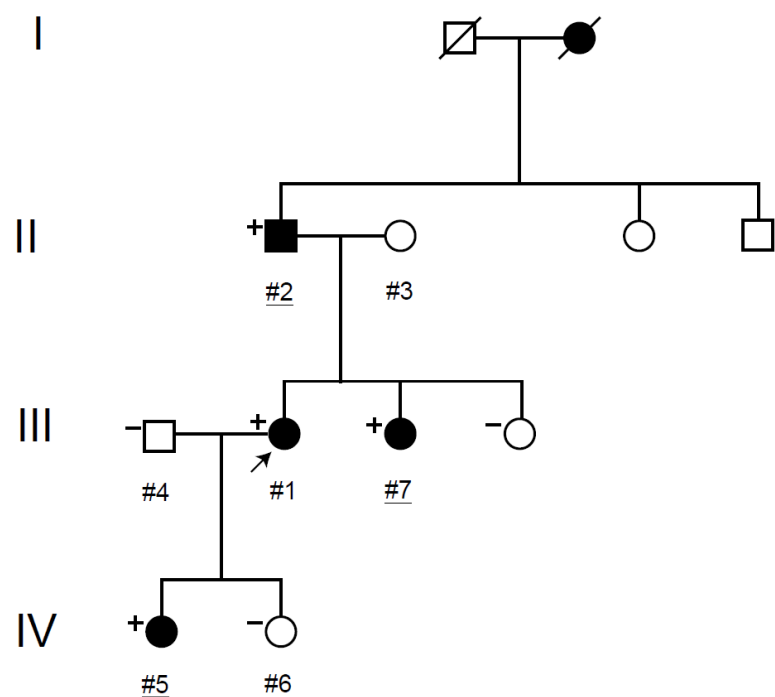
d)
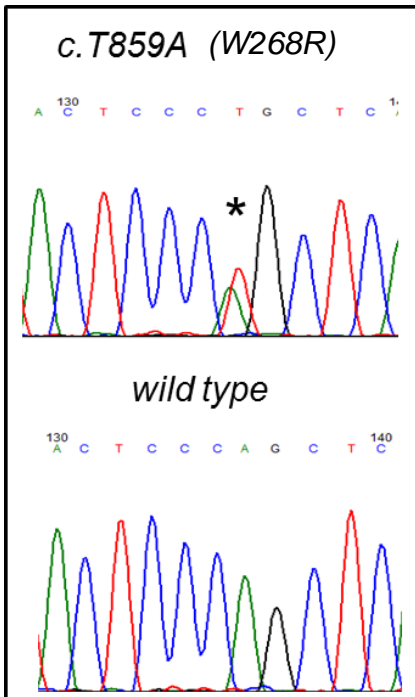
Figure 1

Figure 2

Figure 3

a)

b)

FACAS patient **Healthy control**

c)

Figure 4

Figure 5

a)

Before treatment	After 30 min.	After 90 min.

b)

Icatibant 30 mg

Icatibant 30 mg

Figure 6

Figure 7