A transcriptomics study of hereditary angioedema attacks

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Background: Hereditary angioedema (HAE) caused by C1-inhibitor deficiency is a lifelong illness characterized by recurrent acute attacks of localized skin or mucosal edema. Activation of the kallikrein/bradykinin pathway at the endothelial cell level has a relevant pathogenetic role in acute HAE attacks. Moreover, other pathways are involved given the variable clinical expression of the disease in different patients.
Objective: We sought to explore the involvement of other putative genes in edema formation.
Methods: We performed a PBMC microarray gene expression analysis on RNA isolated from patients with HAE during an acute attack and compared them with the transcriptomic profile of the same patients in the remission phase.
Results: Gene expression analysis identified 23 genes significantly modulated during acute attacks that are involved primarily in the natural killer cell signaling and leukocyte extravasation signaling pathways. Gene set enrichment analysis showed a significant activation of relevant biological processes, such as response to external stimuli and protein processing ($q < 0.05$), suggesting involvement of PBMCs during acute HAE attacks. Upregulation of 2 genes, those encoding adrenomedullin and cellular receptor for urokinase plasminogen activator (uPAR), which occurs during an acute attack, was confirmed in PBMCs of 20 additional patients with HAE by using real-time PCR. Finally, *in vitro* studies demonstrated the involvement of uPAR in the generation of bradykinin and endothelial leakage.
Conclusions: Our study demonstrates the increase in levels of adrenomedullin and uPAR in PBMCs during an acute HAE attack. Activation of these genes usually involved in regulation of vascular tone and in inflammatory response might have a pathogenic role by amplifying bradykinin production and edema formation in patients with HAE. (J Allergy Clin Immunol 2018;142:883-91.)

Key words: *Hereditary angioedema, C1 inhibitor deficiency, peripheral blood mononuclear cells, genes, acute attacks, vascular permeability, plasmin*

Hereditary angioedema (HAE; OMIM #106100) is a potentially life-threating rare disease with autosomal dominant transmission[1,2] caused by reduced antigenic levels (type I) or activity (type II) of C1 inhibitor protein (C1-INH), which is caused by a mutation in the C1 inhibitor gene (*C1INH*, SERPING1; OMIM #606860).[3,4] Other forms of HAE with normal C1-INH levels have been classified.[5-7] Some of these patients have HAE with normal C1-INH levels and mutation in the Factor XII gene; in others the genetic defect is still unknown, and inheritance is derived from symptom segregation.[8] In one family a mutation in the angiopoietin 1 gene has been shown recently to segregate with angioedema symptoms.[9] Severe episodes of edema localized at the subcutaneous and mucosal layers characterize acute attacks of HAE, which can be extremely debilitating, particularly if the airways are affected.[10] The symptoms are caused by local leakage of fluids from the capillaries as a result of uncontrolled activation of the plasma contact system and generation of vasoactive mediators.[5,11]

C1-INH is a serine protease inhibitor that acts as a modulator of different pathways, such as coagulation and fibrinolytic cascades and the complement and contact-kinin systems.[12-14] During HAE attacks, uncontrolled activation of these pathways is enhanced as a result of endothelial cell activation[15] and generates factors that increase vascular permeability, provoking edema and inflammation.[16] It is now well established that bradykinin is the principal mediator of symptoms of acute attacks of angioedema and that local trauma or emotional stress can represent triggering factors for an acute attack.[17-20] However, the molecular events that culminate with the release of bradykinin and generation of tissue edema are not completely understood.[21,22]

Different evidence[23,24] reports that biomarkers for coagulation system activation (Factor XII) and fibrinolytic system activation (plasmin) are functionally linked to bradykinin production,[25] which is associated with endothelial cell activation.[26]

Finally, data from the literature suggest that leukocytes can be involved in the pathogenesis of edema formation,[27] and cytokines are actively released in patients with HAE.[28] Circulating and tissue-infiltrating leukocytes can migrate at the cutaneous and mucosal levels, where they can produce C1-INH and other

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Supported by the Telethon Foundation (Telethon grant no. GGP08223; to G.C., S.C., and M.C.).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication July 21, 2017; revised February 20, 2018; accepted for publication March 28, 2018.

Available online May 4, 2018.

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0091-6749/$36.00

https://doi.org/10.1016/j.jaci.2018.03.016

Abbreviations used
ADM: Adrenomedullin
C1-INH: C1 inhibitor protein
FC: Fold change
FDR: False discovery rate
GSEA: Gene set enrichment analysis
HAE: Hereditary angioedema
HAE-A: HAE caused by C1-INH deficiency during the acute attack
HAE-R: HAE caused by C1-INH deficiency during the remission phase
HS: Healthy subject
uPA: Urokinase plasminogen activator
uPAR: Cellular receptor for urokinase plasminogen activator

complement factors and can potentially contribute to edema formation.[29]

In this study we analyzed the transcriptomic profile of patients during an acute HAE attack in comparison with the same patients in the remission phase while also comparing patients with HAE with healthy subjects (HSs) to identify genes that might have pathogenetic relevance in patients with HAE.

METHODS

An extended description of the materials and methods used in this study is provided in the Methods section in this article's Online Repository at www.jacionline.org.

Patient recruitment

We studied 8 patients with HAE caused by C1-INH deficiency during the remission phase (HAE-R patients; free of attacks for ≥1 month) and during the acute attack (HAE-A patients) involving oral submucosal tissues in 1 patient, subcutaneous tissue in 4 patients, and the abdomen in 3 patients (Table I). Blood samples were collected in PAXgene Blood RNA Tubes (Becton Dickinson, Milan, Italy) within 5 hours from the beginning of symptoms. All patients provided written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of Milan, "Luigi Sacco" Hospital.

Laboratory measurements

The following measurements were performed in nonactivated plasma: C1-INH activity was measured by using a chromogenic assay (Technochrom C1-inhibitor; Techno-clone GmbH, Vienna, Austria), and C1-INH antigen was measured by using radial immunodiffusion (OR-Partigen, Behring, Marburg, Germany; Table II).

RNA extraction

Total RNA was isolated by using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland), according to the manufacturer's instructions. RNA purity and integrity were verified by using lab-on-chip technology (Agilent 2100 Bioanalyzer; Agilent, Palo Alto, Calif).

Gene expression profiling

Transcriptomic data were generated by using the HumanHT-12 v3 Expression BeadChip (Release 38; Illumina, San Diego, Calif), according to the manufacturer's instructions. Illumina arrays were then scanned with the HiScanSQ.

Microarray statistical analyses were performed with GeneSpring GX 11.0 software (Agilent). Identification of genes differentially expressed between HAE-A and HAE-R patients was carried out with the false discovery rate (FDR) method of Benjamini-Hochberg,[30] and gene probe sets were filtered based on FDR (adjusted P value with multiple testing on 1000 permutations) and fold change (FC).[31] Moreover, gene set enrichment analysis (GSEA) was performed in pairwise comparisons of HAE-A versus HAE-R patients.[30]

To assess biological relationships among differently regulated genes, we used the Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, Calif; http://www.ingenuity.com).

The Illumina microarray data are Minimum Information About a Microarray Experiment (MIAME) compliant, and raw data have been deposited in the database of the European Bioinformatics Institute (EMBL-EBI) and are accessible through Experiment ArrayExpress accession (E-MTAB-5897).

cDNA synthesis and quantitative real-time PCR analysis

RT-PCR was performed on total RNA samples (n = 20) by using an ABI Prism 7900HT Sequence detection system (Applied Biosystems, Foster City, Calif). RNA was reverse transcribed into cDNA by using a cDNA synthesis kit (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany). RT-PCR amplification reactions were performed with the QuantiTect Primer Assay and the QuantiFast SYBR Green PCR Mix (Qiagen).

Cell culture

Jurkat T-cell lines (ATCC, Manassas, Va) were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mmol/L glutamine at 37°C in a 5% CO_2 atmosphere and 100 μg/mL penicillin and streptomycin (Sigma-Aldrich, St Louis, Mo).

Primary human umbilical vein endothelial cells were isolated and cultured on bovine gelatin (Sigma-Aldrich) in human endothelial medium supplemented with 10% FBS and 100 μg/mL penicillin and streptomycin (Invitrogen, Carlsbad, Calif) at 37°C in a 5% CO_2 atmosphere.

Bradykinin ELISA

Measurement of bradykinin levels in cell lysates and cell-culture supernatants was assessed by using a human bradykinin ELISA kit (Cloud-Clone, Katy, Tex), according to the manufacturer's instructions. Cells seeded in 6-well plates at a density of 2×10^6 cells/well were preincubated with 10 and 20 μg/mL anti–cellular receptor for urokinase plasminogen activator (uPAR) antibody (Immunological Sciences, Rome, Italy) to neutralize the urokinase plasminogen activator (uPA)–uPAR interaction for 1 hour and then treated with 100 nmol/L uPA (Urokinasi; Hospira, Napoli, Italy) for 15 minutes. Cell lysates and their supernatants were collected subsequently. Bradykinin concentrations were measured at 450 nm and calculated from a standard curve.

Endothelial leakage

The *in vitro* endothelial leakage assay was performed, as previously described.[32] Briefly, human umbilical vein endothelial cells were seeded on a gelatin-coated 24-well-plate transwell (Costar; Corning, Corning, NY) and grown until confluence. Medium for the experiment was supplemented with 10% normal human serum, anti-uPAR antibody was added to endothelial cells at 10 and 20 μg/mL for 15 minutes, and plasminogen was activated with 100 nmol/L uPA for 30 minutes. Then the fluorescein isothiocyanate–BSA that leaked into the lower chamber was evaluated.

Statistical analysis and bioinformatics

For the remaining experimental analysis, we analyzed data using Prism statistical software (GraphPad, La Jolla, Calif). All data were expressed as means ± SDs of data obtained from at least 3 independent experiments. Statistical analysis was carried out with 2-tailed paired and unpaired Student t tests and 1-way ANOVA and Tukey *post hoc* tests. P values of less than .05 were considered statistically significant.

TABLE I. Demographic characteristics of patients with HAE included in the study, gene defects, and type of symptoms during attacks

Patient code	Sex	Age (y)	Site of attack	*SERPING1* transcript mutation
BT	F	48	Oral submucosal tissues	NM_000062.2: c.1481G>T
DG	F	62	Abdomen	NM_000062.2:c.1029+84G>A
MG	F	68	Subcutaneous tissue	NM_000062.2:c.1475T>A
PS	F	48	Abdomen	NM_000062.2:c.1269_1270insA
FF	F	78	Abdomen	NM_000062.2:c.1269_1270insA
CM	M	18	Subcutaneous tissue	NM_000062.2:c.508T>C
LM	M	16	Subcutaneous tissue	large deletion
AC	F	39	Subcutaneous tissue	NM_000062.2:c.-99dup

F, Female; *M*, male.

TABLE II. Measurements of complement parameter markers in 8 HSs, 8 HAE-A patients, and 8 HAE-R patients

Laboratory tests	HSs (n = 8)	HAE during remission phase (HAE-R, n = 8)	HAE during acute attack (HAE-A, n = 8)
C1-INH function (%)	75.9	10.8	4.1
C1-INH antigen (%)	103	26.9	21.0

RESULTS

Differences in gene expression during an acute HAE attack

In the first step of the study, to identify genes/pathways specifically modulated during acute attacks, we compared the whole-genome gene expression profiles of PBMCs from patients with HAE collected during the remission phase (HAE-R patients) with samples from the same patients during the acute attack (HAE-A patients). We identified 23 genes significantly modulated during the acute attack with an FDR of less than 0.05 and an FC of greater than 1.5 (Table III). Expression of these genes was upregulated during acute attacks, and principal component analysis (Fig 1, *A*) showed that these 23 genes were well discriminated during the acute attack and the remission phase.

Then, to determine whether there was a coordinated expression or "enrichment" in a set of functionally related genes in patients with HAE-A, we performed a GSEA. This computational method allowed us to determine whether an *a priori* defined set of genes (involved in a specific pathway or biological process) shows statistically significant concordant differences between PBMCs from HAE-A patients and HAE-R patients. We identified 10 gene sets, each identifying a specific biological process that was modulated significantly ($q < 0.05$) during acute attacks (Table IV). Specifically, sets of genes upregulated during the acute attack were associated mostly with response to external stimulus and protein processing ($q < 0.05$). Among genes contributing to the response to external stimuli, we found *PLAUR*, the gene encoding uPAR, and adrenomedullin (*ADM*; see Table E1 in this article's Online Repository at www.jacionline.org). These 2 genes are factors involved in regulation of vascular tone and could be involved during an acute HAE attack.

Pathway analysis

We performed a pathway analysis to determine global characterization of the biological functions overrepresented by the sets of genes modulated during the acute attack. Analysis revealed that genes identified during the acute attack were involved primarily in the natural killer cell signaling and leukocyte extravasation signaling canonical pathways, as indicated in Fig 1, *B*. In particular, the top-ranked network included several genes encoding for regulators of these pathways (score, 24.8 associated genes; $P < .01$; Fig 1, *C*). Analysis of this network revealed a central role of genes important for regulation of vascular tone. The *ADM* gene codes for a pre-pro-hormone, which is cleaved to form a potent hypotensive and vasodilator agent. The dysferlin *(DYSF)* gene codes for a key calcium ion sensor involved in the Ca^{2+}-triggered synaptic vesicle–plasma membrane fusions and in muscle contraction. Finally, the Fc fragment of IgG receptor III gene *(FCGR3)* codes for a receptor of the Fc region of immunoglobulin γ that binds IgG complexes and mediates antibody-dependent cellular cytotoxicity and other antibody-dependent responses, such as phagocytosis.

Differences in gene expression between patients with HAE and HSs

The second step of the study was to perform whole-genome gene expression analysis using microarray technology to identify genes specifically expressed or modulated in patients with the disease. For this purpose, we compared the genomic profile of PBMCs from HAE-R patients, HAE-A patients, and HSs.

Using ANOVA with the Tukey Honestly Significant Difference *post hoc* test, we found 603 genes to be significantly modulated in patients with HAE compared with HSs (see Table E2 in this article's Online Repository at www.jacionline.org). Principal component analysis showed that these genes could well distinguish the 3 analyzed sample classes (Fig 2, *A*). Most of the identified genes had more different levels of expression than HSs, either during the attack or the remission phase (Fig 2, *B*). Then we performed a GSEA comparing HAE-R patients and HSs to identify the biological processes that could be directly involved in HAE pathogenesis because HAE-R patients are in a physiologic condition similar to that of HSs. The analysis revealed that cell matrix adhesion, cell substrate adhesion, and G protein signaling coupled to cyclic AMP nucleotide second messenger gene sets were the most represented processes differentially regulated in the remission phase of patients with HAE (Table V).

TABLE III. The most significant genes (probes; FDR < 0.05 and FC > 1.5) differentially regulated during the acute HAE attack

Symbol	Entrez gene name	Entrez gene ID	FC
FCGR3B	Fc fragment Of IgG, low affinity IIIb, receptor (CD16b)	2,215	1.5255
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	634	1.5577
NCF1C	Neutrophil cytosolic factor 1C pseudogene	654,817	1.5198
HSH2D	Hematopoietic SH2 domain containing	84,941	1.5723
IL1RN	IL-1 receptor antagonist	3,557	1.6564
ISG20	Interferon-stimulated exonuclease gene 20	3,669	1.5261
HIST2H2AA4	Histone cluster 2, H2aa4	723,790	1.5875
TNFSF13B	TNF superfamily member 13b	10,673	1.5530
IL1RN	IL-1 receptor antagonist	3,557	1.7110
ADM	Adrenomedullin	133	1.5872
HIST2H2AA3	Histone cluster 2, H2aa3	8,337	1.6390
LRG1	Leucine-rich alpha-2-glycoprotein 1	116,844	1.6178
HIST2H2AC	Histone cluster 2 H2A family member c	8,338	1.5242
PADI4	Peptidyl arginine deiminase 4	23,569	1.5031
NCF1	Neutrophil cytosolic factor 1	653,361	1.5944
LPAR2	Lysophosphatidic acid receptor 2	9,170	1.5020
MMP9	Matrix metallopeptidase 9	4,318	1.6025
DYSF	Dysferlin	8,291	1.5576
SLC16A3	Solute carrier family 16 (monocarboxylate transporter), member 3	9,123	1.5833
LILRA5	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	353,514	1.6076
LILRA5	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	353,514	1.5303
FFAR2	Free fatty acid receptor 2	2,867	1.6071
TNFAIP6	TNF-α–induced protein 6	7,130	1.5243

A further pathway analysis of genes modulated in HAE-R patients compared with HSs revealed the most significant biological processes involved primarily in the inflammatory response and immunologic disease canonical pathways, as indicated in Fig E1 in this article's Online Repository at www.jacionline.org.

In particular, 53 genes belonged to the category of inflammatory response (P = 2.73E-07), and 51 genes belonged to the category of immunologic disease (P = 8.26E-09, Table VI). Surprisingly, among genes involved in these biological functions, we found again *ADM* and *PLAUR*, which were significantly downregulated in HAE-R patients compared with HSs, whereas granzyme and perforin-1 expression was upregulated. Moreover, also granzyme B signaling and vascular endothelial growth factor signaling pathways have been found to be significantly modulated in HAE-R patients compared with HSs (see Fig E2 in this article's Online Repository at www.jacionline.org).

Differential expression of *ADM* and *PLAUR* at the transcriptional level

Two of the genes identified by using microarray analysis were of great importance for disease pathogenesis because they are involved not only in regulation of vascular tone and the inflammatory response but also in bradykinin-forming mechanisms controlled by C1-INH. Therefore to confirm gene expression modulation of these 2 genes, we performed quantitative RT-PCR for *ADM* and *PLAUR* in an independent set of 20 patients with HAE with the same clinical and demographic characteristics of those used for microarray experiments. We found that *ADM* and *PLAUR* gene expression was significantly greater during the acute attack episode compared with expression in the same subject in the remission phase (Fig 3, *A* and *B*). These results were in line with those obtained by using the gene expression array.

Blocking the uPA-uPAR interaction alters the bradykinin pathway

The mechanism of action of uPAR in the production of bradykinin was examined. We neutralized uPA-uPAR interaction with a blocking anti-uPAR antibody both at 10 and 20 μg/mL for 1 hour, and then we activated the plasminogen with 100 nmol/L uPA for 15 minutes. Finally, we measured T-cell bradykinin production. Treatment with anti-uPAR antibody attenuated bradykinin production induced by uPA in a dose-dependent manner both in the cell lysate (P = .0002 at 10 μg/mL and P < .0001 at 20 μg/mL; Fig 3, *C*) and in the supernatant (P = .01 at 10 μg/mL and P = .001 at 20 μg/mL; Fig 3, *D*). Anti-uPAR–treated cells showed a significant reduction in bradykinin production also in the control medium without uPA stimulation (see Fig E3 in this article's Online Repository at www.jacionline.org).

Blocking uPA-uPAR interaction alters endothelial leakage

The role of uPAR in endothelial leakage was evaluated by using an *in vitro* assay, as previously described.[32] Anti-uPAR antibody was added to the endothelial cells at 10 and 20 μg/mL for 15 minutes, and then we activated the plasminogen with 100 nmol/L uPA for 30 minutes. Then the fluorescein isothiocyanate–BSA leaked into the lower chamber was evaluated. Treatment with anti-uPAR antibody inhibited the endothelial leakage induced by uPA (Fig 3, *E*).

DISCUSSION

The first finding emerging from our analysis conducted on PBMCs isolated from patients with HAE is that the genes identified during acute attacks were involved primarily in the natural killer cell signaling and leukocyte extravasation signaling

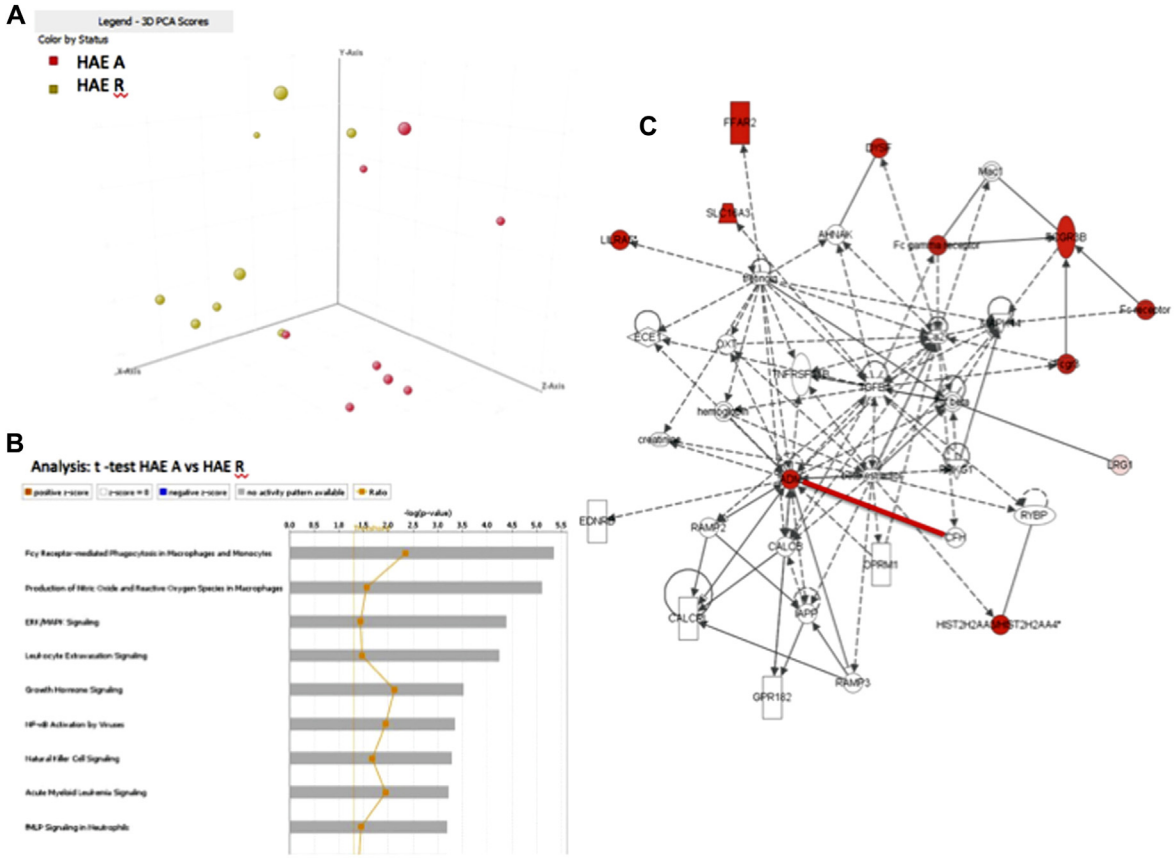

FIG 1. Differences in gene expression during the acute HAE attacks caused by C1-INH deficiency and functional analysis of the top selected genes identified by means of microarray during the acute attack. **A,** Principal component analysis shows that the gene expression profile is different between HAE-A and HAE-R patients. **B,** The most representative canonical pathways dysregulated during acute attacks are involved mainly in natural killer cell signaling and leukocyte extravasation signaling. The *graph* shows category scores; *threshold* indicates the minimum significance level (scored as –log[*P* value] from the Fisher exact test, which is set here to 1.25). *Ratio* (differential yellow line and markers) refers to the number of molecules from the data set that map to the pathway listed divided by the total number of molecules that map to the canonical pathway from within the Ingenuity Pathway Analysis (IPA) knowledge base. **C,** The network has been algorithmically constructed by using IPA software based on the functional and biological connectivity of genes. Genes are symbolized graphically as nodes, and the biological relationships between genes are symbolized as edges. *Red nodes* represent genes containing identified variants; *empty nodes* are biologically linked to the studied genes based on evidence in the literature. Analysis of this top-ranked network (score, 24.8; $P < .01$) reveals the presence of *ADM*, a gene important for regulation of vascular tone. The *red line* evidences the interaction between complement factor H and *ADM*.

TABLE IV. The most represented processes ($q < 0.05$) identified by using the GSEA during the acute HAE attack

Gene set	Total denes	Genes found	*P* value (HAE-A patients vs HAE-R patients)	*q* Value (HAE-A patients vs HAE-R patients)	ES (HAE-A patients vs HAE-R patients)	NES (HAE-A patients vs HAE-R patients)
REGULATION_OF_SECRETION	40	15	0	0.039	0.619	1.836
NEGATIVE_REGULATION_OF_CELLULAR_ BIOSYNTHETIC_PROCESS	28	15	0	0.047	0.683	1.872
PROTEIN_SECRETION	32	17	0	0.017	0.671	2.009
EPIDERMIS_DEVELOPMENT	70	15	.018	0.037	0.665	1.808
PROTEIN_PROCESSING	48	23	0	0.035	0.649	1.821
RESPONSE_TO_EXTERNAL_STIMULUS	306	137	0	0.026	0.519	1.913
PROTEIN_AMINO_ACID_PHOSPHORYLATION	267	132	0	0.041	0.526	1.825
NEGATIVE_REGULATION_OF_BIOSYNTHETIC_ PROCESS	29	16	0	0.031	0.650	1.817
ECTODERM_DEVELOPMENT	79	17	.018	0.035	0.664	1.870
KEGG_SYSTEMIC_LUPUS_ERYTHEMATOSUS	140	58	0	0.100	0.591	1.924

The response to external stimulus gene set built-in genes important for the structure or regulation of extracellular matrix *(PLAUR)* and for regulation of vascular tone *(ADM)* is shown.

ES, Enrichment score; *NES*, normalized enrichment score.

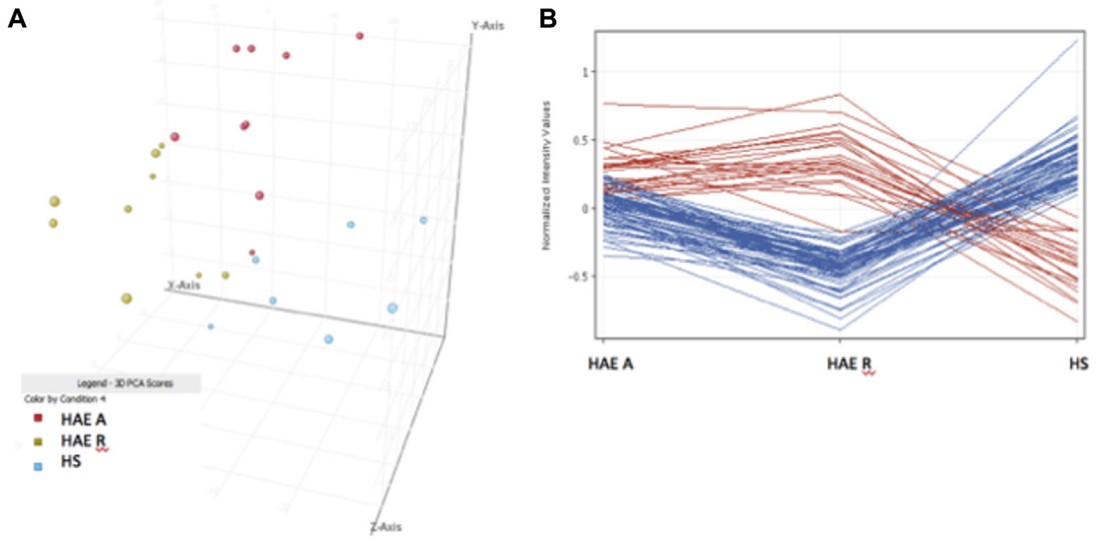

FIG 2. Differences in gene expression between patients with HAE and HSs. **A,** Principal component analysis shows that the gene expression profile is different among the 3 analyzed sample classes. **B,** The profile plot represents gene expression patterns at an FC of 1.5 or greater relative at 90 of 603 genes upregulated and downregulated between patients with HAE and HSs. Upregulated genes are shown in red, and downregulated genes are shown in blue.

TABLE V. The most represented processes ($q < 0.05$) identified by using the GSEA comparing HAE-R patients and HSs

Gene set	Total genes	Genes found	*P* value (HAE-R patients vs HSs)	*q* Value (HAE-R patients vs HSs)	ES (HAE-R patients vs HSs)	NES (HAE-R patients vs HSs)
CELL_SUBSTRATE_ADHESION	38	16	0	0.013	−0.697	−1.941
G_PROTEIN_SIGNALING_COUPLED_TO_CAMP_NUCLEOTIDE_SECOND_MESSENGER	64	20	0	0.018	−0.551	−1.833
CELL_MATRIX_ADHESION	37	15	0	0.007	−0.71	−1.931

ES, Enrichment score; *NES*, normalized enrichment score.

TABLE VI. The most representative canonical pathways dysregulated in HAE-R patients compared with HSs identified by using ANOVA and Tukey tests

Categories	Diseases or functions annotation	*P* value	Activation *z* score	Genes	Molecules
Inflammatory response, organismal injury, and abnormalities	Inflammation of organ	2.73E-07	0.879	***ADM***, *AHNAK, ALOX12, BCL2L1, BCL6, BMX, C5AR2, CA4, CCR6, PNE1, CR1, CXCL1, CXCL16, F5, FCGR2B, FGR, FUT7, FYN, GNB1L, GP1BA,* ***GZMB***, *HCK, IFNAR1, IFNGR2, IMPDH1, IRF5, MAPK3, MGAM, NCF4, NFATC3, NFKBID, NLRP12, NLRX1, NOTCH1, OSM, PGLYRP1,* ***PLAUR***, *PREX1,* ***PRF1***, *PRKCD, SELL, SEMA4A, SMAD7, SMPD1, STX2, TBCE, TGM3, TLR5, TNFRSF1B, TNFRSF9, TUBB1, UTRN, ZFP36*	53
Immunologic disease	Systemic autoimmune syndrome	8.26E-09	0.296	*ACSL1,* ***ADM***, *ALOX12, ALPL, BCL2L1, BCL6, CASP8, CCR6, CD3E, CDA, CR1, CXCL1, CXCL16, DEPDC5, DNM1L, FCGR2B, GNLY, GP1BA,* ***GZMB***, *HCAR3, HCK, IFNAR1, IFNGR2, IMPDH1, IRF5, KCNJ15, LILRB3, LYZ, MAP4K4, MAPK3, MGAM, MSRA, NCF2, NCF4, NCR3, OSM, P2RY13, PADI2, PADI4, PGLYRP1,* ***PLAUR***, *PRF1, SELL, SIRPB1, SP1, TLR5, TNFRSF1B, TNFRSF9, UCP2, VPS37C, ZFP36*	51

The *ADM* and *PLAUR* genes were built in in both the inflammatory response and immunologic disease categories. Boldface genes are citated in the Results section.

pathways. For the first time, our bioinformatics analysis demonstrated that expression of 2 genes, *ADM* and *PLAUR*, was upregulated during acute attacks when comparing HAE-A versus HAE-R patients and was downregulated in HAE-R patients versus HSs. These genes codify for molecules with vasodilator effects and are involved not only in regulation of vascular tone and the inflammatory response but also in bradykinin-forming mechanisms, the principal mediator of HAE attacks.

Our results shed more light on the role of adaptive immunity in the development of HAE which remained controversial over time. Before us, only López-Lera et al[27] analyzed whole-genome RNA expression of PBMCs in 3 HAE type I families according to the presence of mutations and clinical symptoms. They could not

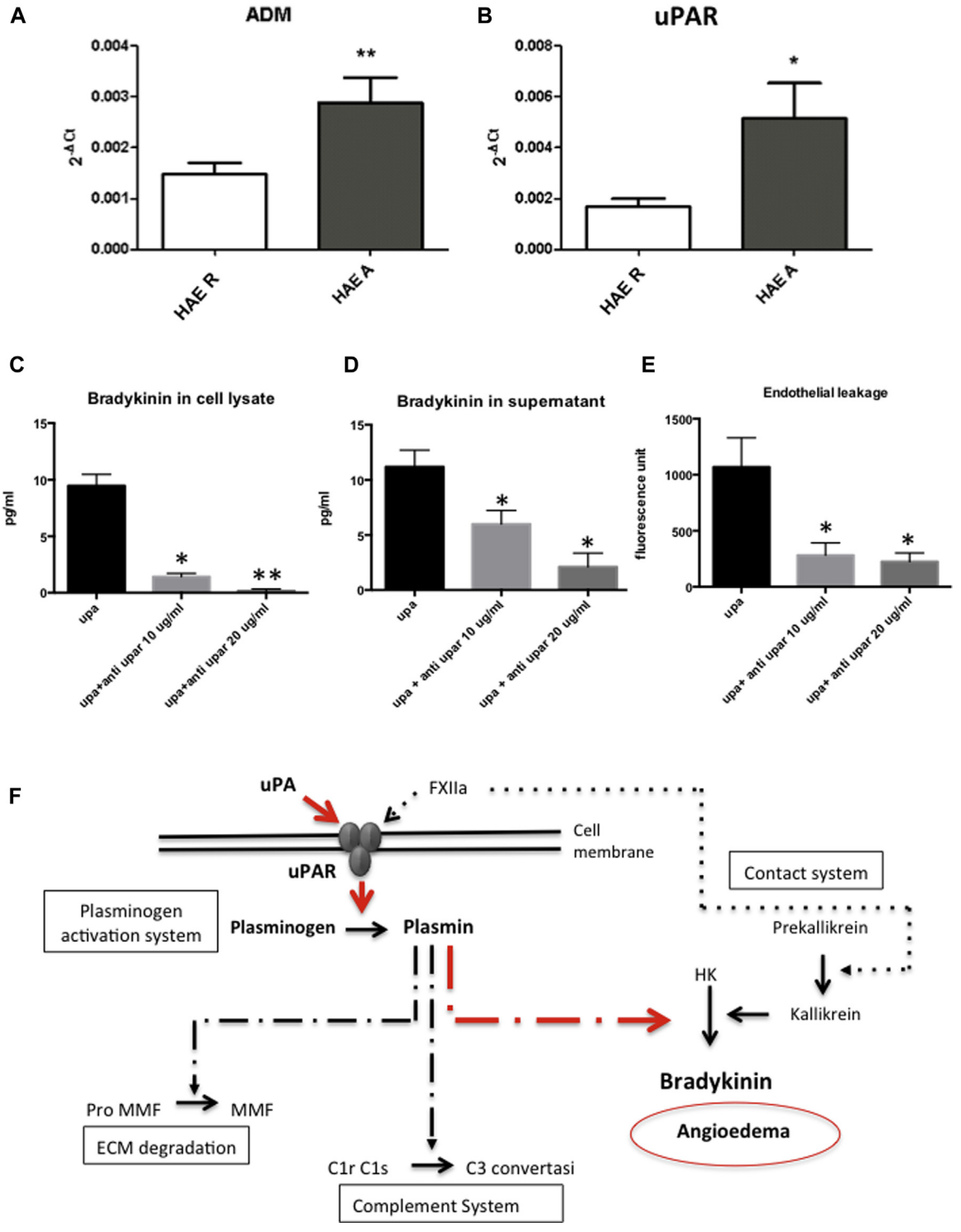

FIG 3. Validation of gene expression levels and functional studies. **A** and **B,** The amount of ADM and uPAR in an independent set of 20 patients with HAE was evaluated by using real-time PCR. *ADM*- and *PLAUR*-normalized gene expression levels are significantly greater during acute disease attacks compared with those in HAE-R patients (*$P < .05$ and **$P < .02$, respectively; P values were obtained by using a paired t test). Histograms represent means ± SDs. **C** and **D,** Treatment with anti-uPAR antibody reduces production of bradykinin in a dose-dependent manner in the Jurkat cell lysate ($P = .0002$ at 10 μg/mL and $P < .0001$ at 20 μg/mL compared with uPA; Fig 3, *C*) and in the supernatant ($P = .01$ at 10 μg/mL and $P = .001$ at 20 μg/mL compared with uPA; Fig 3, *D*). **E,** Treatment with anti-uPAR antibody inhibits the endothelial leakage induced by uPA ($P < .05$ compared with uPA). **F,** Schematic overview of interactions between the contact, complement, and plasminogen activation system with a central role of uPAR in production of bradykinin. *ECM*, Extracellular matrix; *HK*, high-molecular-weight kininogen; *MMP*, matrix metallopeptidase.

find differentially regulated genes in PBMCs of patients with HAE. However, the design of their study was very different and based on comparisons between patients with mutation versus patients without mutation within the analyzed families. Moreover, recent lines of evidence demonstrate that neutrophil and mast cell activation is functionally linked to bradykinin production through contact system activation on endothelial cells.[23] In this regard the results reported by the last evidence and our study support the hypothesis that adaptive immunity function can affect the pathogenesis of HAE.

ADM is a 52-amino-acid vasoactive peptide with vasodilator activity mediated by the cyclic AMP, nitric oxide, and renal prostaglandin systems.[33] ADM acts through a G protein–coupled 7-transmembrane domain receptor, which associates the calcitonin receptor-like receptor. It modulates proteins, namely receptor activity-modifying proteins 2 and 3.[34] ADM could act as an endogenous immunomodulatory factor with predominant anti-inflammatory effects.[35] It is produced in many types of tissue, but the endothelium[36] and monocytes[37] are the principal source, in particular in response to proinflammatory cytokines.[38] ADM reduces endothelial hyperpermeability and is an apoptosis survival factor.[39]

Our microarray data confirmed the study recently published by Kajdácsi e al.[11] They demonstrated that during attacks of HAE caused by C1-INH, the excess of bradykinin that enhanced endothelial cell permeability and activation of endothelial cells was associated with increased levels of several vasoactive peptides, such as ADM, arginine vasopressin, and endothelin-1. In particular, they suggested that cooperation of these vasoactive peptides might be necessary to counterbalance the vasodilatory activity of bradykinin and limit attacks. Recently, Xie et al[37] confirmed these data in patients with acute systemic capillary leak syndrome characterized by abrupt and transient episodes of hypotensive shock and edema caused by plasma leakage into peripheral tissues.

Finally, it is important to note that complement factor H is a serum-binding protein for ADM.[40] The interaction between complement factor H (also known as adrenomedullin-binding protein 1) and ADM has been shown to have possible therapeutic applications in various inflammatory diseases,[41] evidence of which emerged also from our transcriptomic data (Fig 1, *C*). There is plenty of evidence on the reciprocal effects of binding on complement factor H and ADM activity.[42]

Initially, complement activation was proposed in the literature to be the principal pathway involved in the pathogenesis of HAE caused by C1-INH deficiency, but this was later discredited.[22,43-45] As known, the coagulation, fibrinolysis, and plasma kallikrein cascades are interrelated, as shown in Fig 3, *F*, and these cascades are controlled by C1-INH.[46] In the last years, several studies focused on the role of fibrinolysis as a contributor to angioedema,[25,47] and our data are in accordance with this evidence.

uPAR is a glycosylphosphatidylinositol-anchored protein[48] and is able to bind both the pro-form and active form of uPA.[49] Once activated, the primary function of uPA is the conversion of plasminogen to plasmin, a broad-spectrum enzyme capable of widespread extracellular matrix degradation and activation of several pro–matrix metallopeptidases. Several lines of evidence indicate that, in addition to fibrinolysis, the uPA-uPAR system also modulates numerous steps of the inflammatory cascade and influences immune response development.[50]

Inflammation is an adaptive response to damage of vascularized tissues.[51] There is much evidence to suggest that the plasminogen activation system is implicated in regulation of all phases of the inflammatory response but plays a crucial role in leukocyte recruitment to the site of inflammation.[52,53]

uPAR is expressed constitutively in various immunologically active cells, including monocytes, macrophages, and activated T cells, as well as in endothelial cells.[54,55] It is well known that uPAR interacts with components of the bradykinin-forming cascade, but uPAR actually binds high-molecular-weight kininogen best when it has been cleaved[56] and is an important binding protein for Factor XII.[57]

We demonstrated that when we neutralized the uPAR expressed on T cells, bradykinin production was reduced in a dose-dependent manner. These new data demonstrate the involvement of uPAR in production of bradykinin, which is the pivotal mediator of edema formation, showing a plausible additive mechanism of action: uPAR overexpression in HAE-A patients promotes plasminogen activation by uPA, with consequent formation of plasmin finally leading to increased levels of bradykinin and endothelial leakage. This mechanism is in addition to other pathways, such as that of the contact system, in which kallikrein leads to bradykinin production (Fig 3, *F*). Intriguingly, in endothelium, when uPAR is expressed, plasminogen is activated also in the absence of fibrin.[58] Moreover, recently, Joseph et al[59] demonstrated that proinflammatory mediators (IL-1 and TNF-α) and estrogen stimulate release of urokinase from endothelial cells, which can convert plasminogen to plasmin and represent a possible source of plasmin generation. These findings are in agreement with previous observations that in patients deficient in C1-INH the role of plasmin, the key enzyme of the fibrinolytic cascade, appears more prominent.[14,22,24]

We are aware of the limitations of our study. Although we performed a detailed gene expression analysis comparing both HAE-A versus HAE-R patients and patients with HAE versus HSs, we have not investigated ADM and uPAR serum levels because our approved clinical protocol did not consider serum collection.

In conclusion, for the first time, our study suggests that during an acute attack of HAE, expression of 2 genes involved in regulation of vascular tone, *ADM* and *PLAUR*, are upregulated in PBMCs, and the products of these genes could provide a further mechanism involved in bradykinin production and edema formation.

Key message

- **The focus of this study is to identify the genes modulated during acute attacks of HAE that might have a pathogenic relevance in patients with this rare disease.**

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