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RESEARCH

NET Proteomic Profiling Reveals New Pathways Potentially Implicated in Dendritic Cell-Mediated Inflammation in DADA2 Patients

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Abstract

Purpose Adenosine deaminase 2 Deficiency (DADA2) is an autoinflammatory disease characterized by systemic vasculopathy, strokes and mild immunodeficiency. Recently NETosis has been implicated in the pathogenesis of Deficiency of Adenosine Deaminase 2.

To deep investigate the possible effects of NETs on the immune system we characterized proteomic profile of NETs from DADA2 as compared to HD and Polyarteritis Nodosa (PAN) patients. To determine if NETs contain possibly immunogenic antigens we study functional aspects on Dendritic Cells after in vitro stimulation with NETs.

Methods Twenty-three DADA2 patients were enrolled. We analyzed NETosis by Imaging Flow Citometry. We evaluated NETs remnants and DNAse in the plasma samples by ELISA assay whereas DNAse activity by DNA digestion. We used quantitative proteomics approach and network analysis to identify NET proteins and pathways in 6 DADA2, 7 PAN and 7 HD. We analyzed circulating and monocyte-derived dendritic cells by flow cytometry.

Results Neutrophils from DADA2 patients show a significant increased suicidal NETosis. DNAse enzymes were not normal in the level or activity. By proteomic analysis we identified 1356 proteins among which a hundred of proteins were significantly up or down-modulated in DADA2 NETs as compared to normal and disease control NETs in resting condition and after stimulation with PMA, Adenosine and TNFα. DADA2 NETs are significantly more efficient than normal NETs in stimulating patients' monocyte-derived dendritic cells.

Conclusion We identified different pathways significantly modulated in DADA2 NETs versus PAN/HD NETs. This peculiar protein profile could contribute in activating inflammatory pathways in Dendritic cells in DADA2.

Keywords Neutrophil extracellular traps · DADA2 · PAN · Proteomic · Inflammation

Introduction

Deficiency of Adenosine deaminase 2 (DADA2) is a monogenic autoinflammatory disorder presenting with a broad clinical spectrum, including immune dysregulation, inflammatory/vasculitic, and hematological manifestations [1].

Lacunar and ischemic strokes associated with livedo reticularis and a clinical picture consistent with familial polyarteritis nodosa (PAN)-like condition have been originally described [2, 3], despite important differences compared to PAN, particularly in terms of higher incidence of neurological involvement such as stroke and younger age at diagnosis [4, 5]. Most patients with vascular manifestations fulfil the criteria of PAN or cutaneous PAN including angiographic and histopathologic features [2, 7]. DADA2 patients usually carry pathogenic biallelic mutation in ADA2 gene, leading to a decreased ADA2 activity. First descriptions of DADA2 [2, 3] underlined the pivotal role of monocyte/macrophages dysfunction in its physiopathology, but later observations unraveled the remarkable impact of ADA2 deficiency on both innate and adaptive immunity [8–11].

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Neutrophil Extracellular Traps (NETs) dysregulation has been implicated in several autoimmune diseases [13–16], for example as source of autoantigens [18]or carrying immunostimulatory proteins that can activate other immune and stromal cells, induce vascular damage, and promote the induction of the type I interferon pathway and the Nucleotide oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome pathway in other cells [13]. NETs have a direct role in endothelial dysfunction in Systemic Lupus Erithematosus (SLE) patients [19]. NETs increase has been reported in patients experiencing stroke [20], possibly amplifying the thrombosis process [21] and they have been also implicated in the pathogenic process of few monogenic autoinflammatory diseases [22, 23].

Recently, Carmona-Rivera and colleagues [9] investigated the possible involvement of NETosis in DADA2 pathophysiology; ADE was demonstrated to trigger NETs formation, and DADA2 patients' NETs induced a significantly greater production of tumor necrosis factor alpha (TNFα) from M1 macrophages compared to NETs from healthy controls (HDs), potentially linked to differences in the molecular composition of NETs.

Our aim is to further explore the NETosis process in DADA2 patients, quantifying suicidal and vital NETosis induced by several stimuli and evaluating the proper functioning of NETs removal mechanisms. Understanding the biological processes in which NET proteins are implicated may help to elucidate disease molecular mechanisms, by which NETs can act in the disease course. For this reason, we characterized NETs proteins in DADA2 patients through a quantitative proteomics approach and we focused on their effect on maturation and functional impact on Dendritic Cells (DCs) that play a central role in the activation and control of immune responses.

Finally we demonstrated an impairment of NETs degradation mechanisms and identified several pathways associated with DADA2 possibly involved in innate immune dysregulation and inflammation.

Methods

Blood Samples and Clinical Data from Patients and Healthy Donors

HDs and DADA2 patients' blood samples were obtained from the IRCCS Istituto Gaslini and from other Pediatric Rheumatology Italian centers.Informed consent was given by adults or parents or legal guardian for underage patients. DADA2 patients with a genetically confirmed diagnosis and/or with an absent ADA2 activity were enrolled.

Disease controls were identified as patients with childhood Polyarteritis Nodosa (c-PAN), defined according to PRES/PRINTO/EULAR criteria [24], and adults with idiopathic Polyarteritis Nodosa (PAN) recruited by AUSL-IRCCS of Reggio Emilia. Adult patients with PAN met the American College of Rheumatology 1990 classification criteria for PAN [25] and were excluded if there was a history of HBV infection. Therapy controls were identified as Juvenile Idiopathic Arthritis patients treated with etanercept.

Study was approved by the ethical regional and local review board. The main clinical data and laboratory features were reported in Table 1, 2 and Supplementary Table 1 and 2 and 3.

Isolation of Neutrophils and NETs Purification

Untouched neutrophils were isolated from human heparinized whole blood within 2 h, using the MACSxpress® Whole Blood Neutrophil Isolation Kit (Miltenyi Biotech). For NETs purification see supplementary methods.

Imaging Flow Cytometry (IFC): Sample Preparation and Analysis of NETosis

As elsewhere described [26] we employed IFC to quantify 'suicidal' and 'vital' NETosis.

For further details, see the Supplementary Methods.

Plasmatic NETs Remnants, Adenosine, DNase1 and DNase1L3 Quantification

NET remnants in DADA2/HD/PAN plasma samples were evaluated by ELISA assay.

(Cell Death Detection ELISA PLUS, Roche). Adenosine was quantified using Adenosine Assay Kit (Fluorimetric). We measured DNaseI in plasma samples by Human DNASE1 (Deoxyribonuclease-1) ELISA kit, (Biomatik), and DNASE1L3 (deoxyribonuclease 1 like 3) by DNASE1L3 ELISA (LSBioInc) according to the manufacturer's instructions; details are given Supplementary Methods.

DNase Activity

DNase activity was determined with a 1-step assay based on the decrease of fluorescence intensity of degrading Picogreen DNA dye/dsDNA complex in solution; details in Suppl. Methods.

Quantification ROS Production

Total ROS production was analyzed with Neutrophil/Monocyte Respiratory Burst kit (Cayman) according to manufacturer's protocol.

Table 1 Disease course in genetically-confirmed DADA2 patients

Pt	Age at onset/ Sex	Age at diagnosis	Fever	Skin manifestations	Stroke (number)	PNS	Hypertension	Other Symptoms/signs	Biopsy	DADA2 Mutations	Therapies during disease course
1 [1,2]	2y/M	14 years	Yes	Livedo reticularis Subcuteneous nodules	Yes (2)	Peripheral neuropathy	Yes	Growth hormone deficiency, arthralgia, HGG	polyarteritis nodosa (skin)	R312X/ E328D	NSAIDs, Steroids, Thalidomide, Cy, Etanercept
2 [1,2]	9 mo/M	7 years	Yes	Livedo reticularis	Yes (1)	Peripheral neuropathy	Yes	Small bowel invagination, HGG	polyarteritis nodosa (bowel)	R312X/ E328D	NSAIDs, Steroids, Thalidomide, Etanercept
3	6y/F	11y	Yes	Genital ulcers	No	Transient palpebral ptosis	No	Persistent Systemic inflammation, Recurrent MAS episodes, Viral infections	Not done	L183P/L183P	NSAIDs, Steroids, Anakirna, HD IVIG
4 [2,3]	3 mo/F	6y	Yes	Livedo reticularis Subcuteneous nodules	Yes (2)	No	Yes	Myocarditis, HGG, Chondroblastoma	Lobar panniculitis and vasculitis	Homozygous tandem duplication (p.V252Gfs*11)	Steroids, Anakirna, Etanercept
5	20 mo/F	20 years	No	Livedo reticularis Subcuteneous nodules Necrotic ulcers of extremities	Yes (3)	No	Yes	Diarrhoea, abdominal pain, colic ulcerations, HGG, recurrent infections of upper airways	Leucocytoclastic vasculitis (skin), polyarteritis nodosa (bowel)	G47 V/S479P	NSAIDs, Steroids, Cy,MMF, Etanercept
6	2y/M	18 years	No	Livedo reticularis Subcuteneous nodules Necrotic ulcers of extremities	No	Peripheral neuropathy	Yes	Diarrhoea, abdominal pain, arthralgia, HGG, Hypertension related cardiomiopathy	Not done	G47 V/S479P	NSAIDs, Steroids, MMF, Etanercept
7[1,2]	1y/M	12 years	Yes	Livedo reticularis Subcutaneous nodules Ecchimotic lesions	No	No	No	Diarrhoea, Recurrent infection of upper airways	polyarteritis nodosa (skin)	G47 A/ P251L	Steroids, etanercept
8 [1,2]	6 mo/M	8 years	Yes	Livedo reticularis	Yes (2)	Peripheral paresis of the VII cranial nerve Neurosensorial hearing loss	Yes	Myocarditis	Not done	T360 A/ T360 A	NSAIDs, Steroids, Etanercept
9	5y/F	16.5y	Yes	Vasculitic lesions Urticarial rash	Yes(2)	Peripheral neuropathy	Yes	Arthritis, arthralgia, abdominal pain	Not conclusive (superficial)	G47R/G47R	NSAIDs, MPD pulses, Steroids, Etanercept
10	9.5y/F	9.5y	No	No	Yes(1)	No	No	Anemia, systemic inflammation	Not done	Y453 C/ Y453 C	Steroids, etanercept
11	16y/M	52y	Yes	Livedo reticularis Vasculitic lesions and ulcers	Yes (2)	Peripheral neuropathy	Yes	Abdominal pain	Leucocytoclastic vasculitis (skin)	Y220 C/Y220 C	Steroids, Cy, Sulfasalazine, HCQ, MTX, AZA,MMF
12	4y/F	18y	No	Livedo reticularis	Yes(2)	No	No	Systemic inflammation	Not done	L188P/T119 A	Etanercept

Table 1 (continued)

Pt	Age at onset/ Sex	Age at diagnosis	Fever	Skin manifestations	Stroke (number)	PNS	Hypertension	Other Symptoms/signs	Biopsy	DADA2 Mutations	Therapies during disease course
13[4]	6y/M	12y	No	No	No	NO	No	Mild leukopenia, HGG, Splenomegaly Abdominal pain	Not done	L188P/ G383D	Etanercept IVIG
14[5]	14y/F	32y	No	No	No	No	No	Arthralgia, arthritis, Raynaud's phenomenon,, mild leukopenia, mild thrombocytopenia Splenomegaly	Not done	L188P/T187P	Steroids Etanercept
15 [1,2]	5y/F	5y	Yes	Livedo reticularis Necrotic ulcers of extremities Subcutaneous nodules	Yes (1)	Peripheral neuropathy	Yes	Hepatomegaly, splenomegaly, arthralgia, arthritis	polyarteritis nodosa (skin)	T360 A/ T360 A	Steroids, AZA, MTX, Thalidomide
16	6y/M	6.4y	Yes	Livedo reticularis, Subcutaneous nodules	No	No	No	Arthralgia, asthenia, hypertransaminasemia	Not done	G47R/ L249P	Steroids, Etanercept
17	1y/M	13y	Yes	Livedo reticularis	Yes (1)	Neurosensorial hearing loss	Yes (with PRES)	Generalised adenopathy, diarrhoea, oral apthosis, arthralgia, arthritis	Not done	L249P/ P344L	Steroids, etanercept
18 [1,2]	8 mo/M	24y	Yes	Livedo reticularis Purpuric lesions	Yes (1)	Peripheral paresis of the III cranial nerve Optic neuritis	Yes	Hepatomegaly, splenomegaly	polyarteritis nodosa (skin)	G47R/ T360 A	Steroids, AZA, MMF
19 [2]	8y/F	18y	Yes	Livedo reticularis	No	No	No	No	Not done	R45 W/Y453 C	Etanercept
20[1,2]	5y/M	20y	No	Livedo reticularis Necrotic ulcers of extremities	No	No	Yes (with PRES)	Small bowel invagination	polyarteritis nodosa (skin)	L249P/ T360 A	Steroids, Cy, MMF, Infliximab, Etanercept
21	6y/F	10y	Yes	Erythema nodosum Vasculitic lesions	No	No	No	Anemia	Not done	T360 A/T360 A	NSAIDs, Etanercept
22	3y/F	12y	Yes	Livedo reticularis Urticarial rash	No	No	No	No	leucocytoclastic vasculitis (skin)	c.138/144 delG T360 A	Steroids, thalidomide, CSA, MTX, anakirna, adalimumab, Etanercept

Table 1 (continued)

Pt	Age at onset/ Sex	Age at diagnosis	Fever	Skin manifestations	Stroke (number)	PNS	Hypertension	Other Symptoms/signs	Biopsy	DADA2 Mutations	Therapies during disease course
23	12/F	14	Yes	Livedo reticularis Recurrent aphtousis	No	No	Yes	Hepatomegaly, splenomegaly, arthritis, leukopenia, thrombocytopenia, Low IgM, recurrent MAS episodes	Not done	R34 W/P251 =	Anakirna Etanercept

AZA-Azathioprine;Cy- Cyclophosphamide; DADA2- Adenosine deaminase 2 deficiency; HCQ: hydroxychloroquine; HD-IVIG-High dosage Intravenous Immunoglobulin (2 gr/kg); HHG: Hypogammaglobulinemia;MTX-Methotrexate; MMF- Mofetil Mycophenolate; MPD- Metylprednisolone**;** NSAIDs –non steroidal antinflammatory drugs; PAN-Polyarteritis Nodosa, PRES: Posterior Reversible Encephalopathy Syndrome. * DADA2 testing: Genetic test and/or enzymatic activity

1. Caorsi R, Penco F, Grossi A, et al. ADA2 deficiency (DADA2) as an unrecognised cause of early onset polyarteritis nodosa and stroke: A multicentre national study. *Ann Rheum Dis*. 2017;76(10):1648–1656. doi:10.1136/annrheumdis-2016–210802

2. Schena F, Penco F, Volpi S, et al. Dysregulation in B-cell responses and T follicular helper cell function in ADA2 deficiency patients. *Eur J Immunol*. 2021;51(1):206–219. doi:10.1002/eji.202048549

3. Grossi A, Cusano R, Rusmini M, et al. ADA2 deficiency due to a novel structural variation in 22q11.1. *Clin Genet*. 2019;95(6):732–733. doi:10.1111/cge.13518

4. Saettini F, Fazio G, Corti P, et al. Two siblings presenting with novel ADA2 variants, lymphoproliferation, persistence of large granular lymphocytes, and T-cell perturbations. *Clin Immunol*. 2020;218(April):2–4. doi:10.1016/j.clim.2020.108525

5. Dell'Orso G, Grossi A, Penco F, et al. Case Report: Deficiency of Adenosine Deaminase 2 Presenting With Overlapping Features of Autoimmune Lymphoproliferative Syndrome and Bone Marrow Failure. *Front Immunol*. 2021;12(October):1–8. doi:10.3389/fimmu.2021.754029

Table 2 Disease course in PAN patients

Pt	Age at onset/ Sex	Diagnosis/ Age at diagnosis	Fever	Skin manifestations	Stroke (number)	CNS/PNS	Hypertension	Other Symptoms/signs	Biopsy	DADA2 testing*	Therapies during disease course
PAN1	62y/M	PAN/62y	Yes	Livedo reticularis	No	No/Yes	No	None	polyarteritis nodosa (skin)	Negative	Steroids, AZA, CYC, MMF IVIG
PAN2	68y/M	PAN/71y	No	Livedo reticularis Skin ulcers	Yes (1)	No/No	No	None	polyarteritis nodosa (skin)	Negative	Steroids, CSA, MTX, Rituximab, CYC
PAN3	5y/M	c-PAN/7y	Yes	Livedo reticularis Urticarial rash Panniculitis	No	No/No	No	Abdominal pain, arthritis	polyarteritis nodosa (skin)	Negative	MPD, steroids, Anakinra, HCQ, MTX, adalimumab
PAN4	42y/F	PAN/42y	No	Livedo reticularis Skin ulcers	No	No/No	No	None	polyarteritis nodosa (skin)	Negative	Steroids, AZA
PAN5	55y/F	PAN/55y	Yes	No	No	No/No	No	Ischemic abdominal pain	No	Negative	Steroids, CYC, AZA
PAN6	29y/F	PAN/30y	No	Livedo reticularis Skin ulcers	No	No/No	No	None	polyarteritis nodosa (skin)	Negative	Steroids, Rituximab
PAN7	41y/F	PAN/41y	Yes	Livedo reticularis	No	No/No	No	None	polyarteritis nodosa (skin)	Negative	Steroids
PAN8	45y/M	PAN/45y	Yes	No	No	No/No	Yes	None	No	Negative	Steroids, MTX
PAN 9	6y/F	c-PAN/9y	Yes	Skin ulcers Panniculitis Subcuteneous nodules Necrotic ulcers of extremities	No	Yes/Yes	Yes	Arthritis Myalgia Mood disorder Cardiac involvement	polyarteritis nodosa (skin)	Negative	Steroids, CYC, MMF,Etanercept, Tocilizumab, iloprost
PAN 10	10y/F	c-PAN/10y	Yes	Skin ulcers	No	No/No	No	Arthralgia	polyarteritis nodosa (skin)	Not tested	Steroids, thalidomide
PAN 11	8y/F	c-PAN/10y	Yes	Urticarial rash Subcutaneous nodules Oral aftosis	No	No/Yes	No	Arthritis Myositis	polyarteritis nodosa (skin)	Negative	Steroids, MTX
PAN 12	9y/M	c-PAN/11y	Yes	Livedo reticularis Urticarial rash Subcutaneous nodules	No	No/No	No	Uveitis Arthralgia	polyarteritis nodosa (skin)	Negative	Steroids, MTX, AZA, etanercept, adalimumab
PAN 13	8y/M	c-PAN/8y	Yes	Erythematous rash Subcutaneous nodules	No	No/No	No	Arthritis, Myalgia, myositis, epididymitis	polyarteritis nodosa (skin)	Negative	Steroids,CYC, MMF,AZA, thalidomide infliximab, dapsone, adalimumab

Table 2 (continued)

Pt	Age at onset/ Sex	Diagnosis/ Age at diagnosis	Fever	Skin manifestations	Stroke (number)	CNS/PNS	Hypertension	Other Symptoms/signs	Biopsy	DADA2 testing*	Therapies during disease course
PAN 14	7y/F	c-PAN/10y	Yes	Livedo reticularis Erythematous rash Subcutaneous nodules Panniculitis	No	No/Yes	No	Leukopenia, arthralgia, arthritis, myalgia, iridocyclitis	polyarteritis nodosa (skin)	Negative	Steroids,AZA
PAN 15	12y/F	c-PAN/13y	Yes	Vasculitic lesions Panniculitis	No	No/No	No	Arthritis	polyarteritis nodosa (skin)	Negative	Steroids, HD-IVIG, MMF, AZA, CYC, Thalidomide

AZA Azathioprine, *C-PAN* childhood onset Polyarteritis Nodosa, *Cy* Cyclophosphamide, *DADA2* Adenosine deaminase 2 deficiency, *HCQ* hydroxychloroquine, HD-IVIG-High dosage Intravenous Immunoglobulin (2 gr/kg); MTX-Methotrexate; MMF- Mofetil Mycophenolate; MPD- Metylprednisolone; MTX-Metotrexate; NSAIDs –non steroidal antinflammatory drugs; PAN-Polyarteritis Nodosa. * DADA2 testing: Genetic test and/or enzymatic activity

Mass Spectrometry (MS)

Supernatant containing NET proteins (100 uL) was denatured, reduced and alkylated in 100 ul LYSE Buffer (PreOmics). Then proteins were isolated by PAC method [27]. Obtained peptides were analyzed by nano-UHPLC-MS/MS using an Ultimate3000 RSLC coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific Instruments, Bremen, Germany).

Raw data were processed with MaxQuant software [28] version 2.0.3.0. and further processed by IceR [29] with default settings, to reduce the number of missing values.

For Post-translational Modifications (PTMs) analysis raw data were processed with the Open search for PTMs discovery workflow in FragPipe [30]. Details in Suppl. Methods.

Statistics and Bioinformatic Analysis

The Proteins quantification LFQ imputed IceR matrix was statistically evaluated using Perseus software [31] v.1.6.15.0. Overlapping and exclusive elements among the lists of NET proteins were defined by Venn diagrams using jvennweb-based tool [32].

After normalization, differences in proteins expression between HD, PAN, and DADA2 spontaneous or stimuli induced NET were detected with ANOVA test. To reduce the probability of false positive findings deriving from multiple hypothesis testing a permutation-based false discovery rate (FDR) p-value lower than 0.05 was applied, and the artificial within groups variance (S0) was set to 0.1.

Gene ontology (GO) enrichment analysis was conducted on deregulated proteins using the ShinyGO web server (v.0.76.1) [33]. The human proteome served as a reference for the GO enrichment analysis. Details in Suppl. Methods.

Analysis of Protein–Protein Interaction (PPI) Co-Expression Network Modules and Topological Analysis of Co-Expression Network Models

A comparison between PPI and co-expression network models was performed, reconstructing PPI models via STRING [34] and co-expression models using Spearman's correlation. Weighted PPI modules were functionally analyzed to identify enriched pathways, and differentially correlated process and pathway were extracted via statistical tests.

Network topological analysis was conducted using an R script to calculate weighted centrality measures on PPI models, using correlation as edge weight. Nodes with centrality values above the 75 th percentile were identified as hubs or bottlenecks (Details in Suppl. Methods). Subsequently, nodes with betweenness, centroid and bridging values above the 75 th percentile were considered hubs/bottlenecks.

Identification of Conventional DCs (cDCs) and Plasmacytoid DCs (pDCs) in the Peripheral Blood, Monocyte-Derived DC (MODC) Generation and Cytokines Release

cDCs and pDCs were identified and counted by flow cytometry as previously described [35]. MODCs were generated from CD14 + monocytes purified from PBMCs with CD14 microbeads (Miltenyi Biotec) according to manufacturer's instructions. At 7 days cytokines were quantified by flow cytometry bead array (BD) and MODC phenotype analyzed by flow cytometry. The differentiation in MODC was assessed by CD14 down-regulation and CD1a up-regulation. For further details, see Suppl. Methods.

Results

Evidence of Enhanced Suicidal PMA Induced NETosis in DADA2 Patients

The first aim was to assess if neutrophils in DADA2 patients present a NETosis dysregulation.

To this end we modified a quantitative method [26], based on IFC, that simultaneously assess both "suicidal" and "vital" NETosis. An example of analysis template is shown in Supplementary Fig. 1.

Suicidal NETotic cells were characterized as spherical, decondensed, with colocalization of myeloperoxidase (MPO) and nucleus. Vital NETotic cells were characterized by elongated morphology with nucleus condensed and polarized (Fig. 1A, B). The frequency of suicidal NETotic neutrophils stimulated with Phorbol 12-myristate 13-acetate (PMA) was significantly increased in DADA2 patients in comparison with HDs and PAN patients. Conversely the stimulation with Adenosine had a slight effect of induction of suicidal NETosis (Fig. 1A). Moreover PMA stimulated neutrophils from DADA2, showed a trend toward an increased "vital" NETosis, although not significant, again with no effect after ADE stimulation (Fig. 1B).

Indeed we quantified plasmatic Adenosine in DADA2 and HDs and the level was comparable (Fig. 1C). This does not exclude that in the interstitial fluid at the tissue level there may be an accumulation of adenosine that can stimulate neutrophils in the tissue.

To confirm the significant increased NETosis in DADA2 patients we quantified histone-associated-DNA-fragments (mono-and oligonucleosomes), so called NET remnants, by ELISA assay showing a trend toward higher levels in plasma of DADA2 patients ($P = 0.05$), when compared to HDs and PAN patients (Fig. 1D).

DADA2 and PAN Patients Display a Defective DNAse Activity and Increased ROS Production

Since a dysregulated NETosis process can be caused by an enhanced formation of NETs but also by decreased clearance of NETs, we hypothesized that NETs removal mechanisms could be defective in DADA2 patients. The digestion of circulating DNA and of NET-associated DNA is primarily facilitated by DNASE1 and DNASE1L3, which are dedicated to this function [36]. Therefore plasma levels of both enzymes were tested and as shown in Fig. 2A DNASE1 in DADA2 was comparable to normal samples. DNASE1L3 levels in normal donors were generally absent whereas a substantial quantity of circulating enzyme, was present in a significant frequency (60%) of DADA2 samples when compared to HDs/PAN (Fig. 2B). When we measured the plasmatic DNase activity, we found a significant reduced activity in both DADA2 and, to lesser extent, in PAN, when compared to HDs (Fig. 2C).

As shown in Fig. 2D DNAse activity was restored by protein A, suggesting the presence of circulating DNAse inhibitors, that were removed by this protein; this finding rise the hypothesis that the impaired NET degradation in DADA2 could be secondary to a decreased DNase activity due to the presence of a circulating DNase inhibitor.

Suicidal NETosis event [37] is mediated by PAD-4 and promoted by ROS [38], therefore we quantified ROS production and, as expected, it was significantly increased in DADA2 neutrophils (Fig. 2E).

Proteome Profile Shows Peculiar Signature in DADA2 NETs Compared to HD/PAN NETs and Gene Ontology Biological Processes (GOBP) Analysis Highlighted Distinct Pathways and Biological Processes in DADA2

Proteome profile has been analyzed after purification of NETs spontaneously induced (NT) from neutrophils or after separate stimulation with PMA, ADE and TNFα from 7 HDs, 7 PAN and 6 DADA2 patients. A total of 1356 proteins were identified, 986 (72.7%) of which were present in the three clinical groups. Only 26 (1.9%), 61(4.4%) and 53 (3.9%) proteins were exclusively found in the HD, PAN and DADA2 samples, respectively (Fig. 3A). Considering the different stimulations, mimicking the inflammatory environment, we identified in DADA2 NETs 827 (NT), 664 (PMA), 795 (ADE) and 780 (TNFα) proteins found in at least 70% of samples of each group. 540 (54.8%) of which were present in all stimuli. Only 43 (4.4%), 28 (2.8%), 29 (2.9%) and 42 (4.3%) proteins were exclusively found in DADA2 NETs from neutrophils untreated (NT), stimulated with PMA, ADE and TNFα respectively (Fig. 3A).

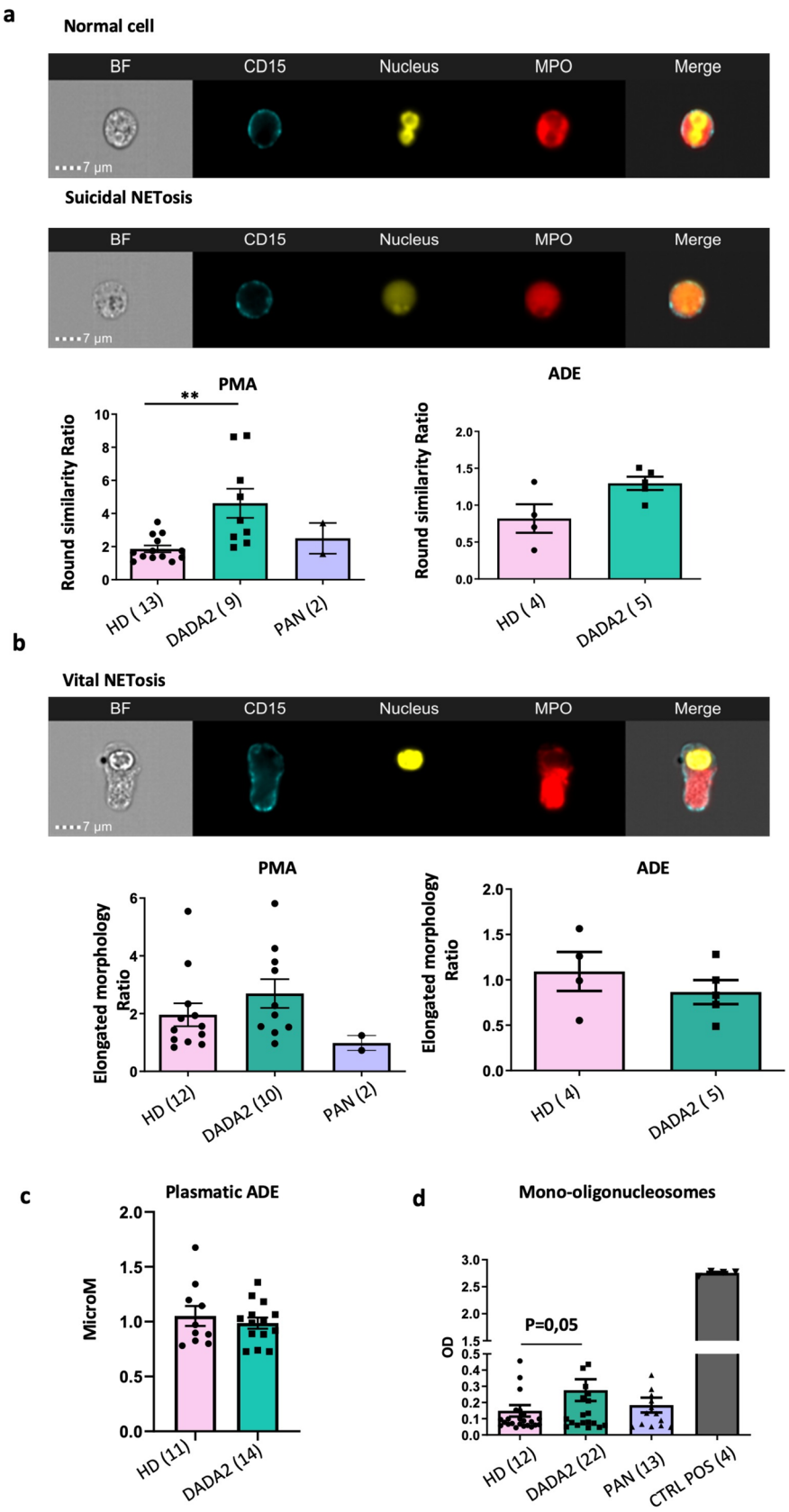


Fig. 1 DADA2 neutrophils show significant exacerbated NETosis. **A** Representative image of a normal and NETotic neutrophil analyzed by IFC. Lower Panel: statistical analysis of cells undergoing suicidal NETosis, represented as Round Similarity Ratio between positive cells in unstimulated and PMA/ADE stimulated samples. **B** Representative image of a vital NETotic neutrophil after PMA/ADE stimulation analyzed by IFC. Lower Panel: statistical analysis of cells undergoing suicidal NETosis, represented as Elongated morphology Ratio between positive cells in unstimulated and PMA/ADE stimulated samples. **C** Quantification of plasmatic Adenosine, **D** Histone-associated-DNA-fragments in DADA2 patients, HDs and PAN patients. Bar plots represent means ± SEM. Significant differences (Mann–Whitney test) are indicated. *P <.05, **P <.01, *** P <.005

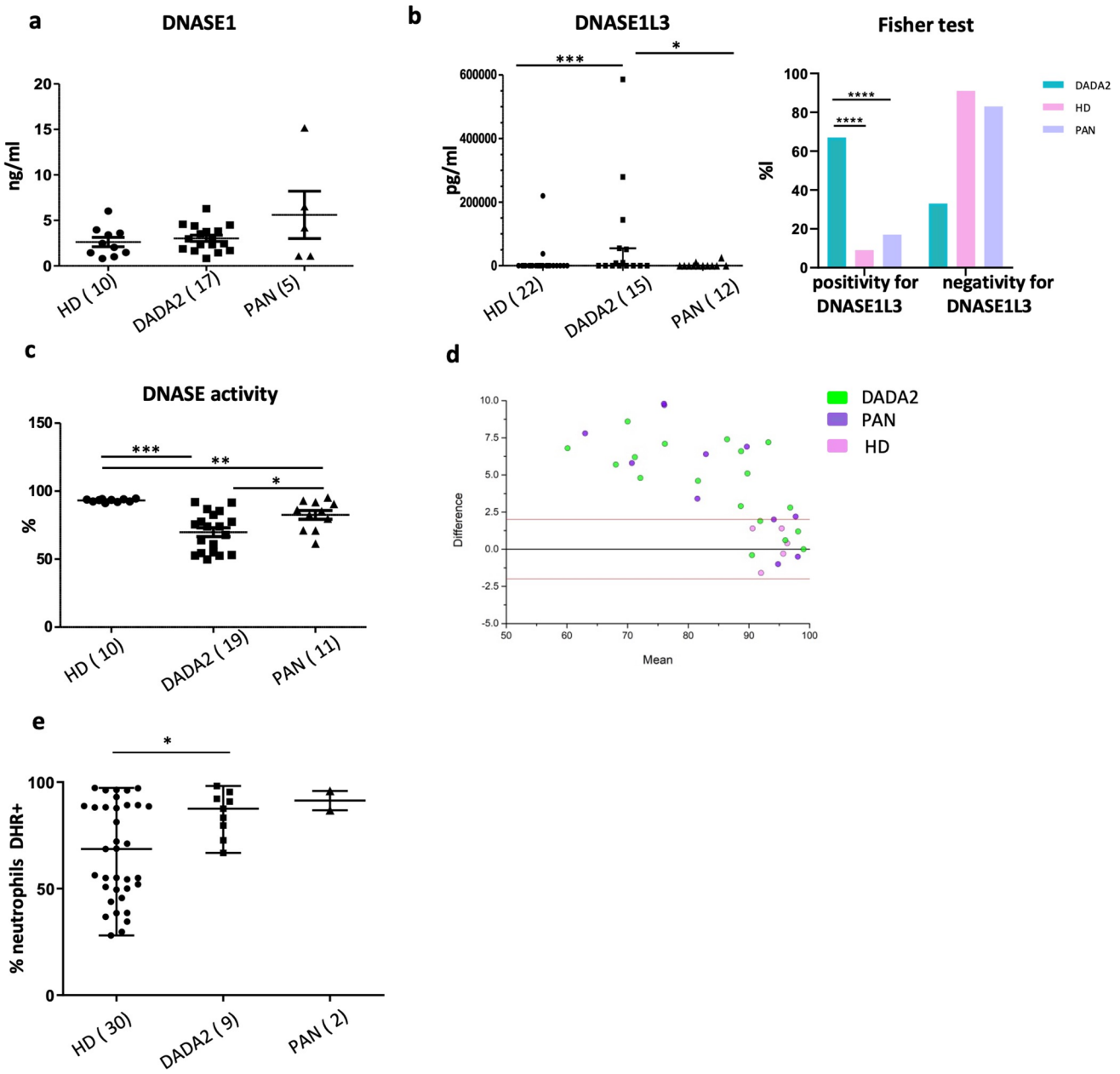


Fig. 2 Dysregulation in NETs removal mechanisms in DADA2 patients and ROS production is increased DADA2 neutrophils. **A** Determination of plasmatic Dnase1 in HD, DADA2 and PAN patients. **B** Determination of DNASE1L3 in HD, DADA2 and PAN plasma patients and frequency of positivity by Fisher test. **C** DNAse activity in HD, DADA2 and PAN. **D** MA plot displaying same samples PAN (purple dots), DADA2 (green dots) and HD (pink dots) analyzed for DNase activity with or without protein A sepharose: outlier data beyond the two red lines have a significant variation. (Non-parametric test Friedman for paired data). **E** Percentage of DHR-123 + positivity gated on cells SSC high/FSC high/; data analyzed by DIVA software and Prism. showing median and range of 30 independent donors, 9 DADA2 and 2 PAN patients

Despite considerable overlapping of protein identity between the three clinical groups and four stimuli, principal component analysis with respect to Component 1 evidenced clear discrimination of two clusters consisting of all DADA2 samples and the other one from the whole of all HDs and PAN samples (Fig. 3B).

Specific protein expression pattern differences in NETs specimens from DADA2 patients were identified by ANOVA test; the expression profile after Z-score normalization of 300 statistically significant proteins in NT (Fig. 3C, Suppl. Table 4) and 278 in PMA (Fig. 3D, Suppl. Table 5) is reported in heatmap diagrams.

Independently of stimulus NET protein expression pattern differs between DADA2, PAN and HDs: in fact we identified the main clusters of proteins whose expression was significantly (FDR < 0.05) upregulated or downregulated with respect to HD/PAN.

A selection of the canonical pathways most significantly associated with the differentially expressed proteins were identified by GOBP analysis.

In the heatmap depicting the proteins significantly modulated by ANOVA test between DADA2/PAN/HD NT, three clusters were highlighted: #296 of the proteins upregulated in DADA2 NETs compared to the other 2 groups, #293 of the proteins downregulated in DADA2 NETs compared to the other 2 groups and #290 of the proteins downregulated in PAN NETs compared to the other 2 groups. One pathway enriched in cluster #296 and highly significant (FDR = 2,7E-14), including 27 proteins was "Myeloid cell activation involved in immune response". They include MNDA (myeloid cell nuclear differentiation antigen), IFI35 (interferon-induced protein 35) and PECAM-1 (Platelet endothelial cell adhesion molecule-1) (Fig. 3E).

The same proteins associated to this pathway have been found significantly upregulated in DADA2 NETs with PMA stimulation in clusters #272 (Fig. 3D) and with TNFα stimulation in cluster #196 (Fig. 4A).

Two pathways enriched in cluster #293 of downregulated proteins in DADA2 NETs, (NT heatmap) and enriched also in cluster #273 of proteins downregulated in DADA2 (PMA heatmap) are "Neutrophil degranulation" and "Neutrophil activation" which include proteins involved in Glucose metabolism. These signatures emerged also in co-expression networks analysis (Fig. 5B, C).

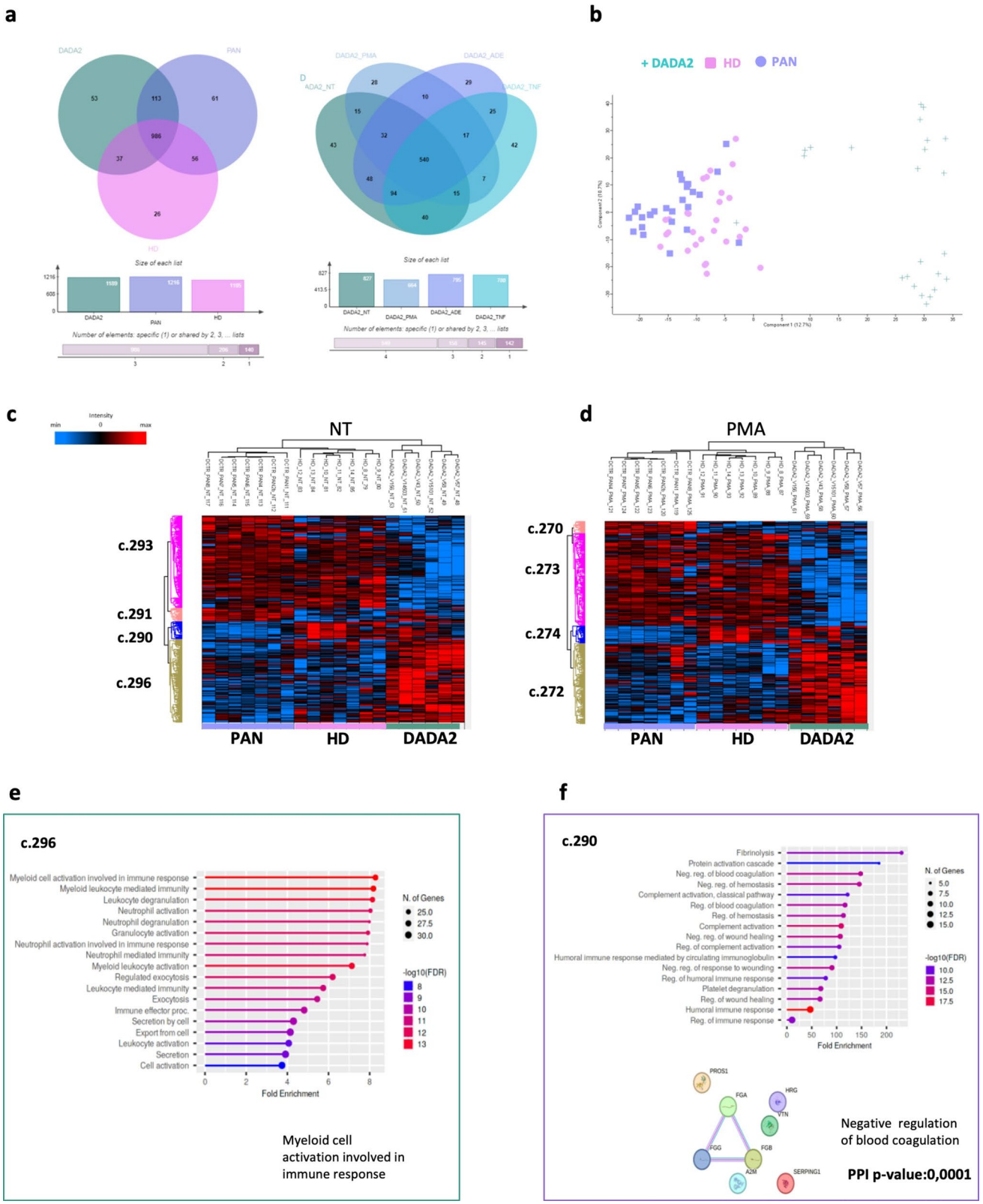
This is probably strictly interconnected with suicidal NETosis because a metabolic shift toward Pentose Phosphate Pathway (PPP) is necessary for NET release [39].

Because the DADA2 patients are on etanercept therapy, we performed an experiment on 3 JIA patients on the same treatment to verify that there was no treatment effect, and Suppl. Figure 2 show that proteins belonging to clusters #296, #272, #196 were not found in the JIA NETs and therefore are not a treatment effect. In previous works [40] the PTMs highlighted modifications of NETs proteins linked to an immunogenic function. So we estimated the percentage of all modifications present in DADA2 NETs versus normal or disease control NETs. In Suppl. Figure 3 the heatmap shows an altered trend in phosphoproteins, but a detailed deep analysis on phosphoproteins results not significant. We identified also the pathways, enriched in the cluster #293 of significantly downregulated proteins in DADA2 NT versus other groups, concerning actin polymerization and depolymerization (Suppl. Table 4); WAS (Wiskott Aldrich syndrome protein) and ARPC1B (Actin related protein 2/3 complex subunit 1 B) belong to this pathway but they have been founded also downregulated in therapy controls JIA NETs (Suppl. Figure 2). This means that these pathways downmodulated could be due to the therapy or to the NETs extrusion process.

If we focus on PAN NETs compared to the other groups, in unstimulated condition, (Fig. 3C), in PMA condition (Fig. 3D), in TNFα condition (Fig. 4A) and in ADE condition (Fig. 4B) significant down-regulated proteins were identified by ANOVA test (Suppl. Table 4, 5, 6, 7). In particular in clusters #290 (Fig. 3C, F), #274 (Fig. 3D), #194 and #288 (Supplementary Fig. 4), the pathways "fibrinolysis" (FDR = 7,29E-12) "negative regulation of blood coagulation" (FDR = 4,32E-14) and "negative regulation of hemostasis" (FDR = 2,07E-13) were enriched. They include downmodulated proteins in PAN NETs PROS1 (Vitamin K-dependent protein S), VTN (vitronectin), FGG, FGA, FGB (Fibrinogen γ α β chains). Other factors are SERPING1 (Plasma Protease C1 Inhibitor), a negative regulator of blood coagulation, whose deficiency leads to a thrombotic effect [41] HRG (Histidine-rich glycoprotein) [42] and A2M (Alfa-2–macroglobulin) that can be seen as both anti- and pro-hemostatic [43] involved in maintaining vascular homeostasis.

TNFα and ADE Stimulate DADA2 Neutrophils to Produce NETs with Specific Proteins Associated with Peculiar Pathways

TNFα is crucial for inflammation in DADA2: for mimicking the inflammatory environment we stimulated neutrophils with TNFα and evaluated NETs proteins: when comparing DADA2 NETs versus HD/PAN NETs by ANOVA test we found a cluster #196 of proteins significantly up regulated in DADA2 respect to the other two groups represented in the heatmap in Fig. 4A (Suppl. Table 6), in which the pathways "cytokine mediated signaling pathway" (FDR = 5,87E-06) and "cellular response to cytokine stimulus" (FDR = 1,51E-06) were enriched. Among the proteins stands out STAT1 (Signal transducer and activator of transcription 1), interferon stimulated genes IFI35 and IFIT3 (interferon induced protein with tetratricopeptide repeats 3).


a
DADA2
PAN
HD
53
113
61
986
37
56
26
Size of each list
1216
608
0
1189
1216
1195
DADA2
PAN
HD
Number of elements: specific (1) or shared by 2, 3, ... lists
3
2
1
DADA2_PMA
DADA2_ADE
DADA2_NT
DADA2_TNF
28
29
10
15
25
32
17
43
42
540
48
7
94
15
40
Size of each list
827
413.5
0
827
664
795
788
DADA2_NT
DADA2_PMA
DADA2_ADE
DADA2_TNF
Number of elements: specific (1) or shared by 2, 3, ... lists
4
3
2
1
b
+ DADA2
HD
PAN
Component 2 (10.7%)
Component 1 (12.7%)
c
Intensity
min
0
max
NT
c.293
c.291
c.290
c.296
PAN
HD
DADA2
d
PMA
c.270
c.273
c.274
c.272
PAN
HD
DADA2
e
c.296
Myeloid cell activation involved in immune response
Myeloid leukocyte mediated immunity
Leukocyte degranulation
Neutrophil activation
Neutrophil degranulation
Granulocyte activation
Neutrophil activation involved in immune response
Neutrophil mediated immunity
Myeloid leukocyte activation
Regulated exocytosis
Leukocyte mediated immunity
Exocytosis
Immune effector proc.
Secretion by cell
Export from cell
Leukocyte activation
Secretion
Cell activation
0
2
4
6
8
Fold Enrichment
N. of Genes
25.0
27.5
30.0
-log10(FDR)
8
9
10
11
12
13
Myeloid cell activation involved in immune response
f
c.290
Fibrinolysis
Protein activation cascade
Neg. reg. of blood coagulation
Neg. reg. of hemostasis
Complement activation, classical pathway
Reg. of blood coagulation
Reg. of hemostasis
Complement activation
Neg. reg. of wound healing
Reg. of complement activation
Humoral immune response mediated by circulating immunoglobulin
Neg. reg. of response to wounding
Reg. of humoral immune response
Platelet degranulation
Reg. of wound healing
Humoral immune response
Reg. of immune response
0
50
100
150
200
Fold Enrichment
N. of Genes
5.0
7.5
10.0
12.5
15.0
-log10(FDR)
10.0
12.5
15.0
17.5
PROS1
FGA
HRG
VTN
FGG
FGB
A2M
SERPING1
Negative regulation of blood coagulation
PPI p-value:0,0001

◂**Fig. 3** Analysis of NETs proteins in DADA2, PAN and HDs: VENN Principal component Analysis (PCA) plot, GO Enrichment of biologically relevant pathways significantly modulated in DADA2 and PAN with respect to control groups. **A** The VENN diagram in the left panel shows the number of identified common and exclusive NET proteins between DADA2, PAN and HDs groups. The VENN diagram in right panel shows NET proteins of DADA2 patients, present at least at 70% in each group, shared or specific for one treatment: NT (untreated), PMA, TNFα, ADE. **B** Principal component Analysis (PCA) plot shows separation among different biological groups on the basis of NET proteins expression profiles. The percentage of the total variation accounted for the first and second component is shown on the x and y axes, respectively. Green crosses, purple squares, and pink circles refer to DADA2, PAN and HD samples respectively. DADA2 and PAN/HD groups form two well separated clusters on Principal Component 1 (PC1). PAN and HD partially cluster on PC2. **C** Heatmap representation shows unsupervised hierarchical clustering analysis of significant NET proteins differentially expressed between DADA2, PAN and HDs groups, identified by ANOVA test (S0 = 0.1 and FDR = 0.05) in untreated (NT) conditions. In the heatmap, each row represents a protein and each column corresponds to a sample. Normalized Z-score protein expression levels are indicated by a two-color scale ranging from blue (lowest values) to red (highest values) reported in the horizontal bar at the right of the panel. The purple, pink and green bars below the columns distinguish the PAN, HD and DADA2 group respectively. The tree dendrogram at the top of the plot displays the results of the unsupervised hierarchical clustering analysis, placing similar sample/proteome profile values next to each other. The optimal association between the main clusters of NETs proteins identified (indicated by the coloured bars on the left side) and DADA2, PAN and HD groups of samples are shown. **D** Heatmap representation after PMA stimulation. **E** Pathway enrichment analyses and network of the protein clusters #296 (NET proteins significantly upregulated in NT neutrophils of DADA2 group respect to PAN/HD groups) of the heat map shown in panel C. Functional enrichment analysis for GO biological processes were conducted by ShinyGO, and enriched pathways were considered significant if FDR value ≤0.05. The graph shows the top enriched terms. **F** Pathway enrichment analyses of the protein cluster #290 (NET proteins significantly down regulated in NT neutrophils of PAN group respect to DADA2/HD groups) of the heatmap shown in panel C. Functional interaction networks refer to "neg. regulation of blood coagulation", pathway of cluster #290 enrichment

The absence of ADA2 activity leads to an accumulation of ADE in patients' plasma; we therefore treated in vitro neutrophils with ADE. By comparing DADA2 NETs treated with ADE versus other two groups, ANOVA test identified cluster #289 of upregulated proteins in DADA2 (heatmap in Fig. 4B and Suppl. Table 7), in which the pathway "regulation of cysteine-type endopeptidase involved in apoptotic processes" (FDR = 4,27E-06) is enriched. CASP8 (Caspase-8), NLRC4 (NLR family CARD domain-containing protein4), HMGB1 (high mobility group box 1) are associated to this pathway.

Co-Expression Networks Highlight Biological Processes in DADA2 NETs

Following the merging of PPI and co-expression network models, we identified biological processes and pathways whose global correlation significantly changed in pairwise comparisons (Suppl. Table 8–9). In DADA2 NETs (vs HD), we observed an increased correlation of Actin cytoskeleton-related processes, including actin nucleation, actin2/3 complex-mediated actin nucleation and actin polymerization-dependent cell motility (Fig. 5A). Of note, the same processes were also most correlated in DADA2 NETs vs PAN NETs.

As for REACTOME (Fig. 5B), significant correlated pathways in DADA2 NETs included Neutrophil Degranulation [44], the Pentose Phosphate Pathway [45] (PPP), the Negative regulation of NOTCH4 signaling and the Tumor necrosis factor receptor 2 (TNFR2) non-canonical NF-kB pathway [46]. They were most correlated in DADA2 NETs in comparison to both HD/PAN. In this scenario, the PPP was further extracted as most correlated in PMA and TNFα-treated samples compared to NT, whereas it was down correlated in ADE compared to NT. This supports the hypothesis that the PPP could serve as a link between the absence/presence of ADE and the formation of NETs [39] Finally, glycolysis was found to be more correlated in ADE samples (vs NT) and less correlated in TNFα samples (vs NT), while cori cycle less correlated in those TNFα and ADE treated (Fig. 5C).

With the purposes to identify relevant proteins underlying the investigated phenotypes, in addition to protein quantitation, we processed the HD/DADA2 and PAN NETs weighted network models for extracting topological relevant nodes (Suppl. Table 10). Among the ten best ranked hubs/bottlenecks per condition, in DADA2 vs PAN NETs we noted the presence of different heat shock proteins (Hsp). In particular, DnaJ Hsp Family Member A1 (DNAJA1), Hsp90 Alpha Family Class B Member 1 (HSP90 AB1) and Hsp Family A Member 5 (HSPA5) were found in DADA2, while Hsp Family A Member 9 (HSPA9), and Hsp90 Alpha Family Class A Member 1 (HSP90 AA1) were in PAN. Of note, DADA2 was characterized also by the presence of Superoxide Dismutase 1 (SOD1) [47] and Interferon Gamma Inducible Protein 16 (IFI16) [48]. FGG, FGA, FGB are hub genes in PAN NETs, confirming proteomic data (Fig. 5D).

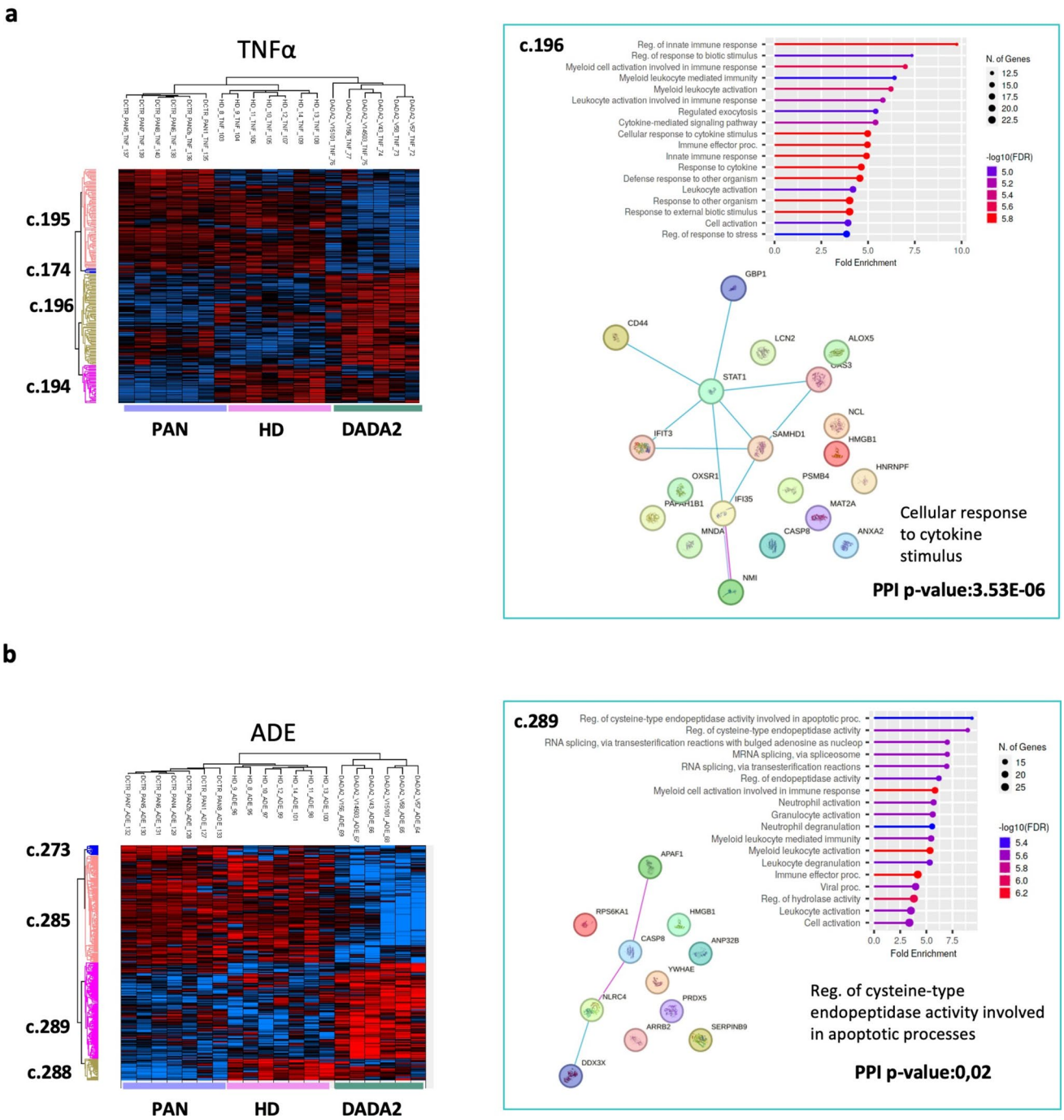


Fig. 4 Hierarchical Clustering, GO Enrichment of biologically relevant pathways upregulated in DADA2 with respect to control groups with TNFα and ADE stimulation. **A** In the left panel the heatmap representation shows unsupervised hierarchical clustering analysis of significant NET proteins differentially expressed between DADA2, PAN and HD groups, identified by ANOVA test (S0 = 0.1 and FDR = 0.05) after TNF stimulation. In the right panel is shown the pathway enrichment analyses of the protein cluster #196 (NET proteins of TNF stimulated neutrophils upregulated in DADA2 respect to HD/PAN). Functional interaction network refers to the "cellular response to cytokine stimulus" pathway of cluster #196 enrichment is shown. **B** In the left panel the heatmap representation shows unsupervised hierarchical clustering analysis of significant NET proteins differentially expressed between DADA2, PAN and HD groups, identified by ANOVA test (S0 = 0.1 and FDR = 0.05) after ADE stimulation. In the right panel is shown the pathway enrichment analyses of the protein cluster #289 (NET proteins of ADE stimulated neutrophils upregulated in DADA2 respect to HD/PAN). Functional interaction network refer to the "Reg. of cysteine-type endopeptidase activity involved in apoptotic processes" pathway of cluster #289 enrichment. In the right panel the biological processes identified in NETs generated after ADE stimulation is shown

MODCs from DADA2 Patients Differentiate Normally, but PMA Induced NETs from DADA2 Induce Release of pro-Inflammatory Cytokines in DADA2 MODCs

From previous paper [9] it is known that NETs stimulate macrophages to secrete TNFα; in the present study we unravelled as upregulated in DADA2 NETs several proteins belonging pathways such as myeloid cell activation. Dendritic cells are professional component of myeloid cells that orchestrate immune reactions and, when activated secrete various cytokines. So we wondered if cDCs and pDCs were normally distributed in DADA2 patients and if NETs can have a direct effect on the MODCs maturation and cytokines secretion. DADA2 patients have a normal distribution of cDCs and pDCs in peripheral blood: in Fig. 6A the percentage and absolute numbers of respectively 10 and 9 DADA2 patients are shown. The in vitro differentiation of MODCs from DADA2 patients did not differ from HDs: in fact the percentage of MODCs obtained is 60% in both HDs and DADA2 patients (data not shown) and the expression of the activation and maturation markers CD80, CD83, CD86 and HLADR was comparable as also the effect of DADA2 or HD NETs (Fig. 6B).

MODCs from DADA2 patients showed a decreased, although not significant, spontaneous secretion of pro-inflammatory cytokines TNFα, IL6 and IP-10 when compared to MODCs from HDs. This event could be depend on the effect of the in vivo treatment Interestingly the incubation with PMA induced DADA2 NETs, but not HD neither PAN NETs, affected DADA2 MODCs, but not HD MODCs, to significantly upregulate the secretion of TNFα and IL-6. On the other hand, both DADA2 NETs and HD NETs induced a significant upregulation of IP-10 secretion by both DADA2 and HD MODCs (Fig. 6C-D). The production was inhibited by DNAse1 and human recombinant ADA2 to signify a disease-specific NETs effect.

Discussion

In this work we investigated the NETosis process, NETs removal mechanisms in DADA2 patients and the in vitro effect of NET stimulation on MODCs. In line with a previous study, we show that NETosis in DADA2 patients is exacerbated [9] also in remission phase of disease.

We did not verify a significant effect of Adenosine as an inducer of Netosis, probably because we evaluated an early step in the process and by a different method. Moreover, a decreased DNAse activity was also noted, suggesting an accumulation of autoantigens and DNA not properly disposed, which could represent a further trigger for an inflammatory or auto-reactive response. Notably DNAse1L3 activity inversely correlates with anti-DNA autoantibodies response [36].

In line with these results we identified by proteomic approach the proteins overexpressed in DADA2 NETs compared to normal/PAN NETs MNDA [49], IFI35 [50] and IFIT3, that are associated to type 1 IFN axis and IFI16 as hub gene in DADA2.

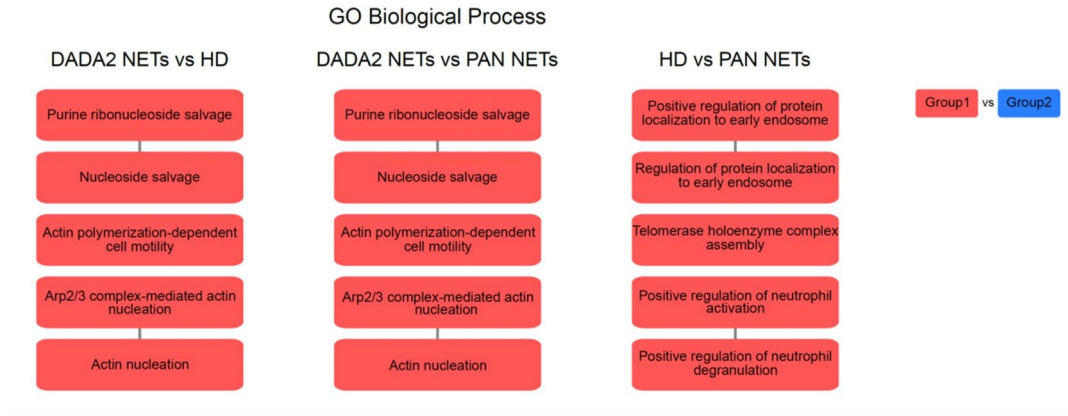
The role of IFN in DADA2 pathogenesis is still controversial. This pathway has been identified as activated signature in DADA2 monocytes [51] and in T cells [52, 53]. The present data are consistent with that published by Belot et al. [54] showing a positive IFN signature associated to neutrophil activation. These observations suggest a possible link between the increased NETosis and the activation of the IFN pathway in DADA2.

Interestingly IFI35 and IFIT3 are also over-expressed in NETs derived from TNFα-stimulated DADA2 neutrophils, suggesting a possible role of TNFα as a trigger the IFN responses and it could be indicating a cross-regulation of this two signaling pathways. In a small cohort of patients, the in vivo treatment with etanercept has been reported to significantly reduce the IFN signature [55], further supporting the possible role of TNFα and NETs in the IFN pathway up-regulation observed in DADA2.

Recent studies [52, 53] identified STAT1 as an important hub for type2 IFN signature in T cells and in T-monocytes cross-talk in DADA2. In the present study the in vitro stimulation of neutrophils with TNFα induces upregulation of STAT1 in DADA2 NETs, suggesting the possibility that neutrophils could also induce IFN response via STAT1 pathway in DADA2 patients, corroborating the possible cross-regulation between TNFα/IFNγ. This finding is consistent with the secretion of IP-10 from monocyte-derived dendritic cells stimulated by DADA2 NETs. IP-10 could have a cascading effect on adaptive immunity in DADA2 patients, in particular on Th1/Th17 differentiation.

This report expand the previous study, focused on the possible role of NETosis in DADA2 [9], providing a new

a



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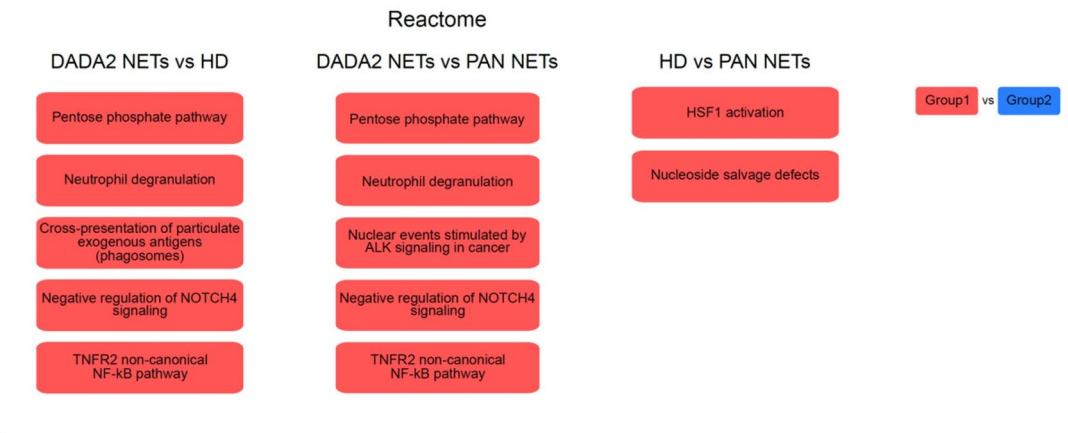

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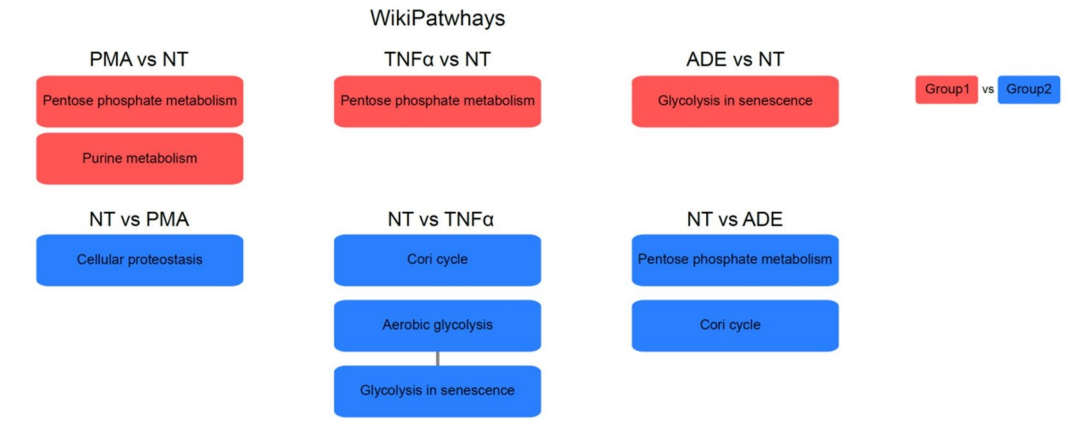

d

HD				DADA2				PAN			
Hub/Bottleneck	Betweenness	Centroids	Bridging	**Hub/Bottleneck**	Betweenness	Centroids	Bridging	**Hub/Bottleneck**	Betweenness	Centroids	Bridging
VCP	87222	0	431,4	**SOD1**	39957	-197	331,1	**CIT**	86454	0	385,6
HNRNPL	30659	-597	206,1	**DNAJA1**	29978	-55	362,4	**HNRNPL**	36456	-273	246,9
HSP90AA1	28745	-135	203,9	**HSP90AB1**	29285	-43	240,9	**YWHAQ**	33328	-157	385,8
BAG3	27687	-403	208,5	**UBC**	28834	-303	168,7	**HSPA9**	33168	-241	366,5
NPM1	27465	-377	177,5	**CLTC**	26609	-301	252,6	**HSPA5**	25073	-293	170
YWHAE	23228	-613	237,9	**VCP**	25881	-413	128,1	**BAG3**	23850	-473	180,4
UBE2M	22993	-613	112,2	**HSPA5**	25200	-369	167,9	**PCBP1**	22687	-795	229,1
CAPZB	22696	-507	105,8	**DHX9**	24661	-171	203,3	**HSP90AA1**	22157	-267	158,2
CALD1	21187	-399	322,6	**RPS3**	23025	-129	180,6	**LRRK2**	18914	-601	65,5
CANX	20387	-585	194	**IFI16**	22335	-119	184,7	**FLNA**	16880	-571	208,1

◂**Fig. 5** Differentially correlated functional modules extracted by processing the protein expression profiles from DADA2 NETs (vs HD NETs and vs PAN NETs).For each comparison, the most relevant terms (FDR < 0.05) are shown. Connected terms share at least 60% of their genes. **A** Gene Ontology (GO) Biological Processes. **B** REACTOME pathways identified by comparing DADA2 NETs vs HD NETs and PAN NETs conditions. **C** Differentially correlated pathways by comparing samples treated with PMA, TNF or ADE (vs NT) are shown; specifically, in red are highlighted pathways up correlated in PMA, TNF or ADE, while in blue are highlighted those most correlated in NT (FDR < 0.05). **D** Best ten-ranked hubs/bottlenecks in HD NETs, DADA2 NETs and PAN NETs. Hubs/bottlenecks were selected based on betweenness, centroid and bridging centralities

evidence on the ability of DADA2 NETs to stimulate the production of pro-inflammatory cytokines by autologous MODCs (Fig. 7) Further studies are needed to elucidate the molecular mechanisms.

Indeed it is conceivable that the mechanisms responsible for the production of TNFα and IL6 stimulated by DADA2 NETs on DADA2 MODCs depend on molecular pathways different from those involved in the production of IP-10. The latter would seem to depend on pathways activated by both NETs and active on MODCs from patients and controls.

Interestingly in DADA2 NETs purified from ADE and TNFα stimulated neutrophils, HMGB1 is upregulated; this alarmin has been localized in NETs of other conditions such as SLE, rheumatoid arthritis and gout [56, 57] suggesting its pathogenic role in the inflammation. Indeed HMGB1 stimulation enhances cytokine (IL-1β, IL-6, TNFα) production in myeloid DCs [58]. This protein could be a good candidate to mediate NET proinflammatory effect by triggering of TLRs or RAGE receptor, but its role needs to be validated. Interestingly it has been implicated in thrombotic effect, neuroinflammation and stroke [21].

ADE stimulated neutrophils from DADA2 patients display significantly abundant CASP8 and NLRC4 in the NETs. Interestingly inflammasome proteins were elevated in NETs present in thrombi of AIS patients. The activation of the CASP8 pathway has been observed in kidney neutrophils to ANCA-Associated Vasculitis, condition in which NETosis play a pathogenic role [59].

Noteworthy, DADA2 NETs upregulated PECAM-1, a molecule expressed mainly in neutrophils and macrophages, that has been found into the brain three days after stroke onset [60]; its overexposure in DADA2 NETs could be implicated in patients neuroinflammation.

Differently from DADA2, PAN NETs downregulate PROS1, VTN, SERPING1, HRG, A2M, proteins involved in coagulation process. Indeed FGG, FGA, FGB are best hubs in PAN NETs. It is known that NETs contribute to the development of thrombosis providing a scaffold for procoagulant effectors consisting of platelets, vWF, TF, extracellular histones, neutrophil serine, proteases, and fibrinogen. Our observation brings to the attention that PAN NETs could be associated with a dysregulation of coagulation process possibly implicated in the peripheral vasculitis or vascular complications of PAN. Indeed an increased risk of venous thromboembolism has been reported in PAN [61].

In conclusion in the present report we identified a number of proteins significantly modulated in DADA2 NETs that could contribute in perpetuate vascular inflammation that distinguishes this condition. We do not know at present whether these proteins are equally implicated in BM disease, but it would be interesting to study NETs from patients with haematologic phenotype.

This work also assumes significance in light of the newly discovered lysosomal deaminase function of ADA2, which acts as a sensor of intracellular DNA [62] Indeed, NETs has been shown in SLE to efficiently trigger activation of pDCs via TLR-9 [63]; now deoxyadenosine-to-deoxyinosine editing on DNA by ADA2 via TLR-9 opens up new scenarios about the patients'DNA modifications, including that contained in NETs; and what cascading activations on cells of innate immunity such as dendritic cells might have.

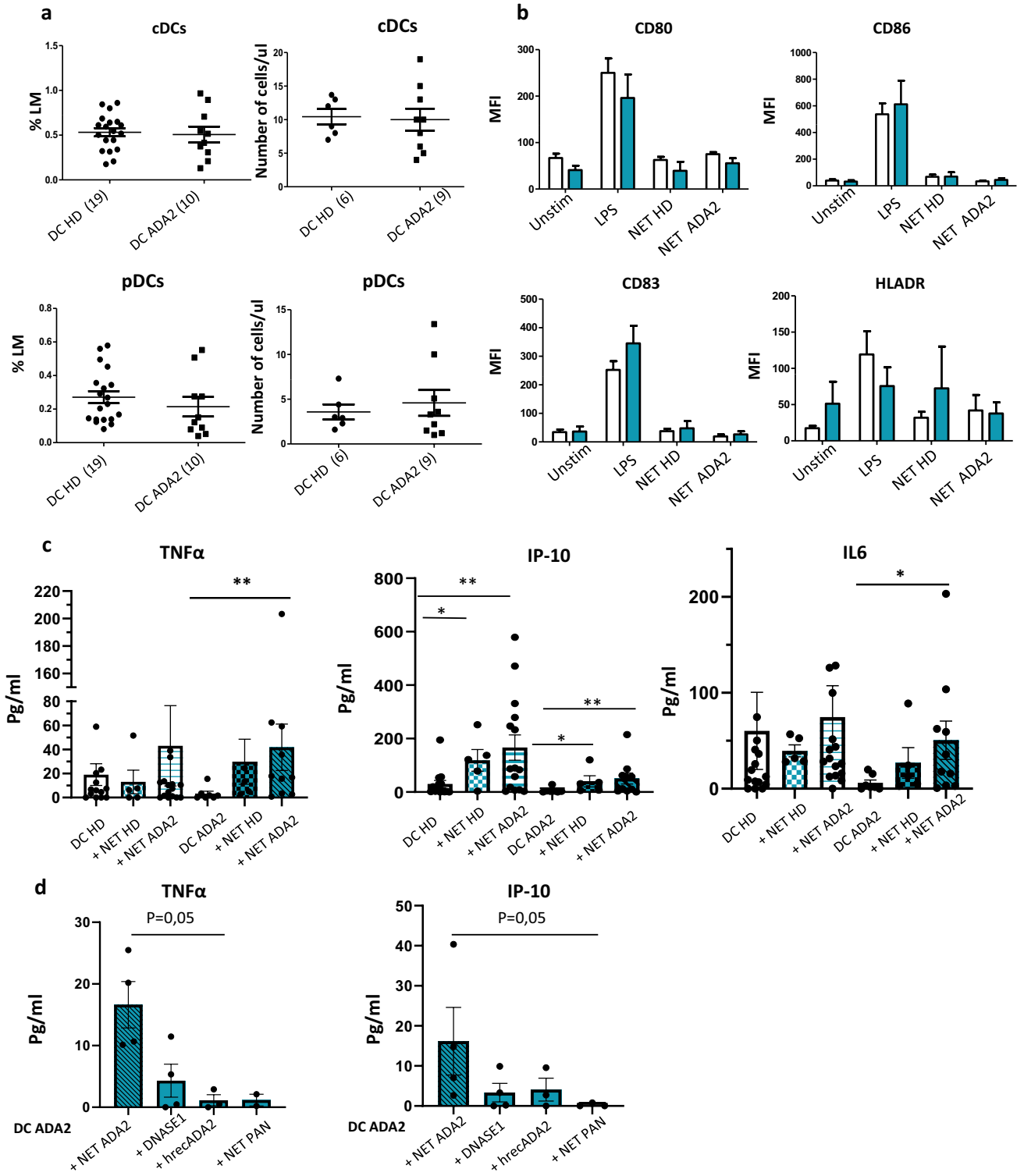

Fig. 6 Distribution of conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs) in DADA2 patients and effect of DADA2 NETs on maturation and cytokines secretion from MODCs. **A** Frequency and absolute count of cDCs (upper panel) and pDCs (lower panel), gated on lympho-monocytes. **B** Expression of activation and maturation markers in MODCs: CD80, CD83, CD86, HLADR. **C** In vitro production of pro-inflammatory cytokines by MODCs from HDs (blank bars) and DADA2 patients (green bar) stimulated with normal NETs or DADA2 patients derived NETs. **D** In vitro production of pro-inflammatory cytokines by MODCs from DADA2 patients stimulated with autologous DADA2 NETs or in presence of DNASE1/hrecADA2 or stimulated with PAN NETs. Data shown represent means ± SEMs. Significant differences (Unpaired t test) are indicated. *P <.05, **P <.01, *** P <.005

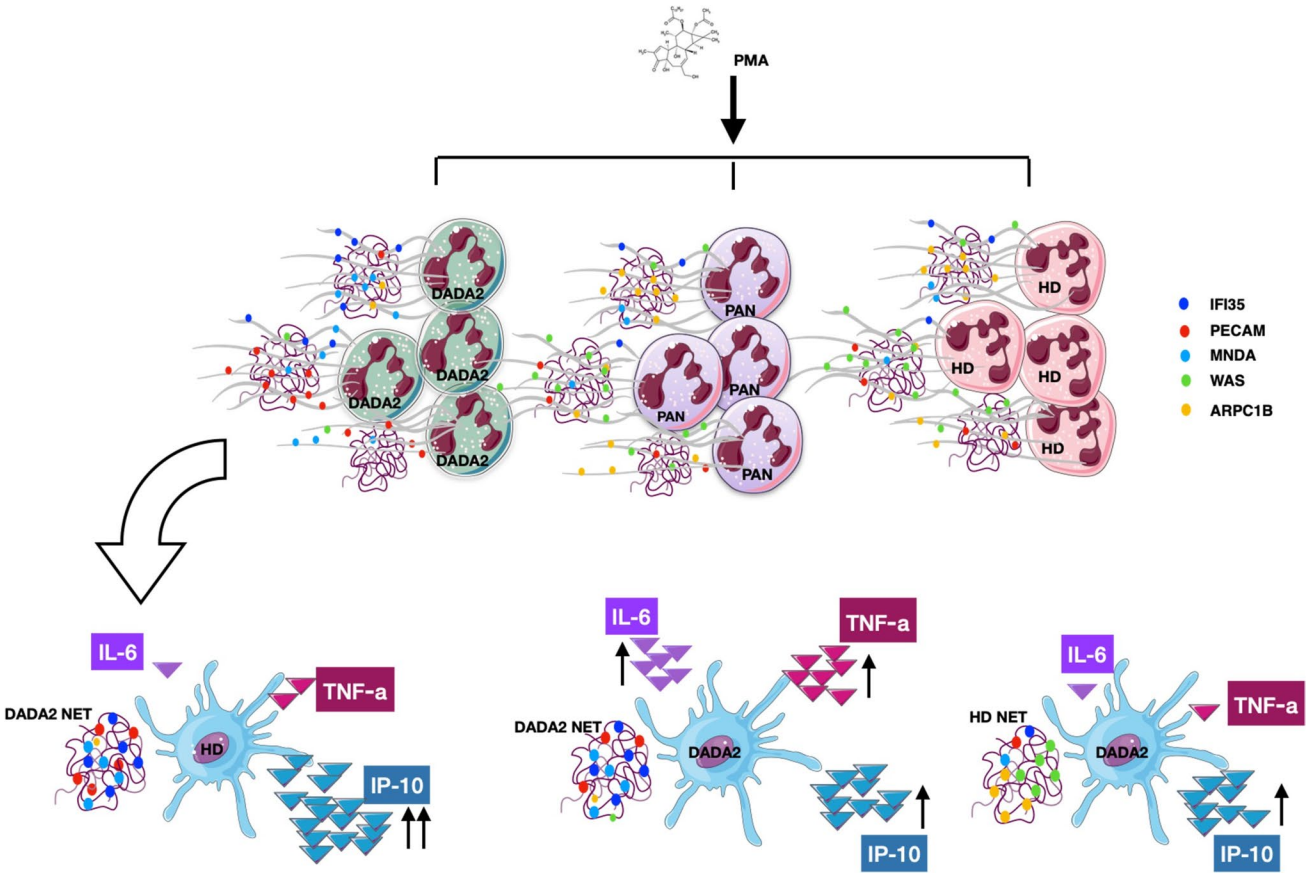

Fig. 7 Graphical abstract of the work

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10875-025-01888-w.

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Authors' Contributions SS: in vitro experiments, data interpretation, manuscript writing, coordination of patients' samples collection

MB: proteins purification, proteomic analysis, elaboration of the manuscript

AB, AC, MB, LP: in vitro experiments and statistical analysis

GDZ: IFC experiments, data analyses and financial support

SB: in vitro experiments and statistical analysis. He is a recipient of a competitive felloship from the PhD program of Experimental medicine, University of Milan, "La Statale"

SV: data interpretation, financial support, critical revision and final approval

RB: design the study, data interpretation, final approval of manuscript

AP: design the study, proteomic analysis and financial support

SC, MB : in vitro experiments, elaboration of manuscript

CS: coordination of patients' samples collection, financial support

RC, AI, MGC and GT: coordination of patients' samples collection, clinical data collection

AL, DDS, PM: network analysis PB: technical support

SDB, DM: data interpretation, critical revision and final approval

MG: design the study, coordination of patients' samples collection, financial support, data interpretation, elaboration of the manuscript and final approval

FS: Design of the study, in vitro experiments and analysis, data interpretation, manuscript writing and final approval.

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Data Availability The full sets of mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [64] partner repository with the dataset identifier PXD055246 (Reviewer account details: Username: reviewer_pxd055246@ebi.ac.uk Password: nFAEDqUGvsmt).

Declarations

Competing interests The authors declare no competing interests.

Authors' disclosures All the authors declare nothing to disclose pertinent to this study.

References

1. Barron KS, et al. The spectrum of the deficiency of adenosine deaminase 2: an observational analysis of a 60 patient cohort. Front Immunol. 2021;12.
2. Navon Elkan P, et al. Mutant adenosine deaminase 2 in a polyarteritis nodosa vasculopathy. N Engl J Med. 2014;370(10):921–31.
3. Zhou Q, et al. Early-onset stroke and vasculopathy associated with mutations in ADA2. N Engl J Med. 2014;370(10):911–20.
4. Schnappauf O, et al. Sequence-based screening of patients with idiopathic polyarteritis nodosa, granulomatosis with polyangiitis, and microscopic polyangiitis for deleterious genetic variants in ADA2. Arthritis Rheumatol. 2021;73(3):512–9.
5. Kasap Cuceoglu M, et al. Systematic review of childhood-onset polyarteritis nodosa and DADA2. Semin Arthritis Rheum. 2021;51(3):559–64.
6. Huang Z, et al. Polyarteritis nodosa and deficiency of adenosine deaminase 2 - shared genealogy, generations apart. Clin Immunol. 2020;215.
7. Sharma A, et al. Deficiency of adenosine deaminase 2 in adults and children: experience from India. Arthritis Rheumatol. 2021;73(2):276–85.
8. Yap JY, et al. Intrinsic defects in B cell development and differentiation, T cell exhaustion and altered unconventional T cell generation characterize human adenosine deaminase type 2 deficiency. J Clin Immunol. 2021;41(8):1915–35.
9. Carmona-Rivera C, et al. Deficiency of adenosine deaminase 2 triggers adenosine-mediated NETosis and TNF production in patients with DADA2. Blood. 2019;134(4):395–406.
10. Lee PY, et al. Mechanisms of vascular inflammation in deficiency of adenosine deaminase 2 (DADA2). Semin Immunopathol. 2022;44(3):269–80.
11. Lee PY. NETing the mechanism of inflammation in DADA2. Blood. 2019;134(4):338–9.
12. Signa S, et al. Adenosine deaminase 2 deficiency (DADA2): a crosstalk between innate and adaptive immunity. Front Immunol. 2022;13.
13. Wigerblad G, Kaplan MJ. Neutrophil extracellular traps in systemic autoimmune and autoinflammatory diseases. Nat Rev Immunol. 2023;23(5):274–88.
14. Liu CL, et al. Specific post-translational histone modifications of neutrophil extracellular traps as immunogens and potential targets of lupus autoantibodies. Arthritis Res Ther. 2012;14(1):R25.
15. Lood C, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. Nat Med. 2016;22(2):146–53.
16. Villanueva E, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory

molecules in systemic lupus erythematosus. J Immunol. 2011;187(1):538–52.
17. Arneth B, Arneth R. Neutrophil extracellular traps (NETs) and vasculitis. Int J Med Sci. 2021;18(7):1532–40.
18. Jariwala MP, Laxer RM. NETosis in rheumatic diseases. Curr Rheumatol Rep. 2021;23(2):9.
19. Carmona-Rivera C, et al. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. Ann Rheum Dis. 2015;74(7):1417–24.
20. Ducroux, C., et al. Thrombus Neutrophil Extracellular Traps Content Impair tPA-InducedThrombolysis in Acute Ischemic Stroke. Stroke. 2018; 49(3): 754–757.
21. Gao X, et al. Neutrophil extracellular traps mediated by platelet microvesicles promote thrombosis and brain injury in acute ischemic stroke. Cell Commun Signal. 2024;22(1):50.
22. Apostolidou E, et al. Neutrophil extracellular traps regulate IL-1beta-mediated inflammation in familial mediterranean fever. Ann Rheum Dis. 2016;75(1):269–77.
23. Mistry P, et al. Dysregulated neutrophil responses and neutrophil extracellular trap formation and degradation in PAPA syndrome. Ann Rheum Dis. 2018;77(12):1825–33.
24. Ozen S, et al. EULAR, PRINTO, PRES criteria for henoch-schonlein purpura, childhood polyarteritis nodosa, childhood wegener granulomatosis and childhood takayasu arteritis: ankara. part II: final classification criteria. Ann Rheum Dis. 2008;69(5):798–806.
25. Lightfoot RW Jr, et al. The american college of rheumatology 1990 criteria for the classification of polyarteritis nodosa. Arthritis Rheum. 1990;33(8):1088–93.
26. Zhao W, et al. A novel image-based quantitative method for the characterization of NETosis. J Immunol Methods. 2015;423:104–10.
27. Batth TS, et al. Protein aggregation capture on microparticles enables multipurpose proteomics sample preparation. Mol Cell Proteomics. 2019;18(5):1027–35.
28. Cox J, and Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 2008;26(12):1367–72.
29. Kalxdorf M, et al. IceR improves proteome coverage and data completeness in global and single-cell proteomics. Nat Commun. 2021;12(1):4787.
30. Yu F, et al. Identification of modified peptides using localization-aware open search. Nat Commun. 2020;11(1):4065.
31. Tyanova S, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods. 2016;13(9):731–40.
32. Bardou P, et al. Jvenn: an interactive venn diagram viewer. BMC Bioinformatics. 2014;15(1):293.
33. Ge SX, et al. ShinyGO: a graphical gene-set enrichment tool for animals and plants. Bioinformatics. 2020;36(8):2628–9.
34. Szklarczyk D, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. Nucleic Acids Res. 2021;49(D1):D605–12.
35. Carenza C, et al. Comprehensive phenotyping of dendritic cells in cancer patients by flow cytometry. Cytometry A. 2021;99(3):218–30.
36. Sisirak V, et al. Digestion of chromatin in apoptotic cell microparticles prevents autoimmunity. Cell. 2016;166(1):88–101.
37. Brinkmann V, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532–5.
38. Sollberger G, et al. Neutrophil extracellular traps: the biology of chromatin externalization. Dev Cell. 2018;44(5):542–53.
39. Azevedo EP, et al. A metabolic shift toward pentose phosphate pathway is necessary for amyloid fibril- and phorbol 12-myristate 13-acetate-induced neutrophil extracellular trap (NET) formation. J Biol Chem. 2015;290(36):22174–83.
40. Bruschi M, et al. Neutrophil extracellular traps protein composition is specific for patients with Lupus nephritis and includes methyl-oxidized alphaenolase (methionine sulfoxide 93). Sci Rep. 2019;9(1):7934.
41. Grover SP, et al. C1 inhibitor deficiency enhances contact pathway-mediated activation of coagulation and venous thrombosis. Blood. 2023;141(19):2390–401.
42. Gao S, Nishibori M. Multiple roles of histidine-rich glycoprotein in vascular homeostasis and angiogenesis. Acta Med Okayama. 2021;75(6):671–5.
43. Lagrange J, et al. Alpha-2-macroglobulin in hemostasis and thrombosis: an underestimated old double-edged sword. J Thromb Haemost. 2022;20(4):806–15.
44. Meier S, et al. Neutrophil degranulation, NETosis and platelet degranulation pathway genes are co-induced in whole blood up to six months before tuberculosis diagnosis. PLoS ONE. 2022;17(12):e0278295.
45. Awasthi D, et al. (2023) Modulations in human neutrophil metabolome and S-glutathionylation of glycolytic pathway enzymes during the course of extracellular trap formation. Biochim Biophys Acta Mol Basis Dis. 1869;1:166581.
46. Neuenfeldt F, et al. Inflammation induces pro-NETotic neutrophils via TNFR2 signaling. Cell Rep. 2022;39(3):110710.
47. Talal S, et al. Neutrophil degranulation and severely impaired extracellular trap formation at the basis of susceptibility to infections of hemodialysis patients. BMC Med. 2022;20(1):364.
48. Thompson MR, et al. Interferon gamma-inducible protein (IFI) 16 transcriptionally regulates type i interferons and other interferon-stimulated genes and controls the interferon response to both DNA and RNA viruses. J Biol Chem. 2014;289(34):23568–81.
49. Gu L, et al. Myeloid cell nuclear differentiation antigen controls the pathogen-stimulated type I interferon cascade in human monocytes by transcriptional regulation of IRF7. Nat Commun. 2022;13(1):14.
50. De Masi R. et al.& IFP35 is a relevant factor in innate immunity, multiple sclerosis, and other chronic inflammatory diseases: A Review. Biology (Basel) 2021;10(12).
51. Watanabe N, et al. Analysis of deficiency of adenosine deaminase 2 pathogenesis based on single-cell RNA sequencing of monocytes. J Leukoc Biol. 2021;110(3):409–24.
52. Wu Z, et al. Single-cell profiling of T lymphocytes in deficiency of adenosine deaminase 2. J Leukoc Biol. 2022;111(2):301–12.
53. Nihira H, et al. Detailed analysis of Japanese patients with adenosine deaminase 2 deficiency reveals characteristic elevation of type II interferon signature and STAT1 hyperactivation. J Allergy Clin Immunol. 2021;148(2):550–62.
54. Belot A, et al. Mutations in CECR1 associated with a neutrophil signature in peripheral blood. Pediatr Rheumatol Online J. 2014;12:44.
55. Insalaco A, et al. Variable clinical phenotypes and relation of interferon signature with disease activity in ADA2 deficiency. J Rheumatol. 2019;46(5):523–6.
56. Castiglioni A, et al. High-mobility group box 1 (HMGB1) as a master regulator of innate immunity. Cell Tissue Res. 2011;343(1):189–99.

57. Mitroulis I, et al. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PLoS ONE. 2011;6(12): e29318.
58. Song X, et al. HMGB1 Activates Myeloid Dendritic Cells by Up-Regulating mTOR Pathway in Systemic Lupus Erythematosus. Front Med (Lausanne). 2021;8: 636188.
59. Hu J, et al. Caspase-8 activation in neutrophils facilitates autoimmune kidney vasculitis through regulating CD4(+) effector memory T cells. Front Immunol. 2022;13:1038134.
60. Winneberger J, et al. Platelet endothelial cell adhesion molecule-1 is a gatekeeper of neutrophil transendothelial migration in ischemic stroke. Brain Behav Immun. 2021;93:277–87.
61. Ungprasert P, et al. Risk of venous thromboembolism among patients with vasculitis: a systematic review and meta-analysis. Clin Rheumatol. 2016;35(11):2741–7.
62. Greiner-Tollersrud OK, et al. ADA2 is a lysosomal deoxyadenosine deaminase acting on DNA involved in regulating TLR9-mediated immune sensing of DNA. Cell Rep. 2024;43(11): 114899.
63. Lande R. et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med. 2011;3(73), 73ra19.
64. Vizcaino JA, et al. Update of the PRIDE database and its related tools. Nucleic Acids Res. 2016;44(22):11033.

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