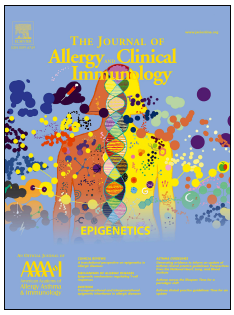
Journal Pre-proof



Genotype and functional correlates of disease phenotype in deficiency of adenosine deaminase 2 (DADA2)

Pui Y. Lee, MD PhD, Erinn S. Kellner, MD, Yuelong Huang, PhD, Elissa Furutani, MD, Zhengping Huang, Wayne Bainter, MMsc, Mohammed F. Alosaimi, MD, Kelsey Stafstrom, MS, Craig D. Platt, MD PhD, Tali Stauber, MD, Somech Raz, MD PhD, Irit Tirosh, MD, Aaron Weiss, DO, Michael B. Jordan, MD, Christa Krupski, DO MPH, Despina Eleftheriou, MBBS, MRCPCH, PhD, Paul Brogan, MBChB, FRCPCH, PhD, Ali Sobh, MD, Zeina Baz, MD, Gerard Lefranc, PhD, Carla Irani, MD MsCE, Sara S. Kilic, MD, Rasha El-Owaidy, MD PhD, M.R. Lokeshwar, MD DCH, Pallavi Pimpale, MD, Raju Khubchandani, MD, Eugene P. Chambers, MD, Janet Chou, MD, Raif S. Geha, MD, Peter A. Nigrovic, MD, Qing Zhou, PhD

PII: S0091-6749(20)30030-0

DOI: https://doi.org/10.1016/j.jaci.2019.12.908

Reference: YMAI 14360

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 30 October 2019

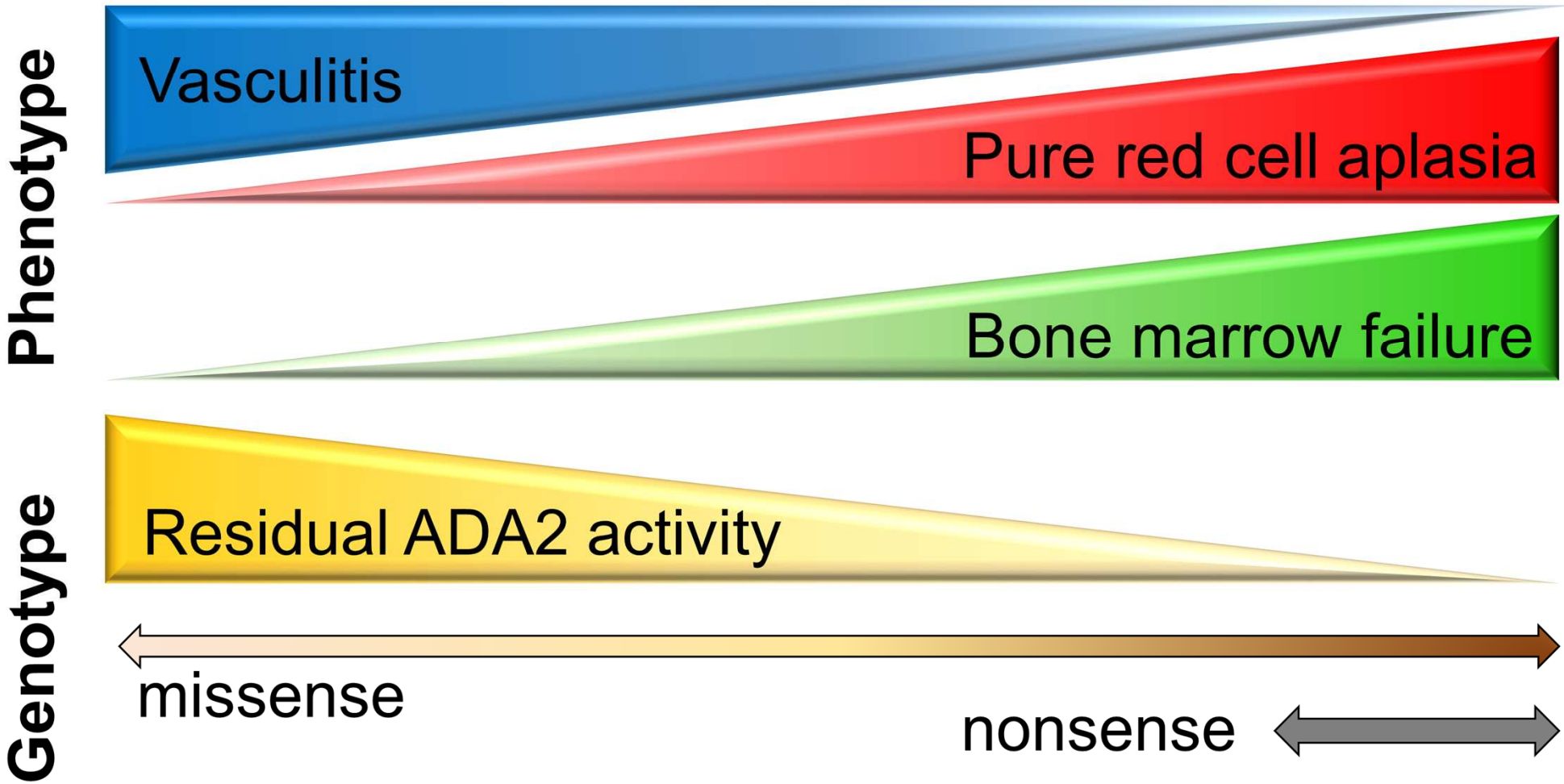
Revised Date: 7 December 2019

Accepted Date: 27 December 2019

Please cite this article as: Lee PY, Kellner ES, Huang Y, Furutani E, Huang Z, Bainter W, Alosaimi MF, Stafstrom K, Platt CD, Stauber T, Raz S, Tirosh I, Weiss A, Jordan MB, Krupski C, Eleftheriou D, Brogan P, Sobh A, Baz Z, Lefranc G, Irani C, Kilic SS, El-Owaidy R, Lokeshwar MR, Pimpale P, Khubchandani R, Chambers EP, Chou J, Geha RS, Nigrovic PA, Zhou Q, Genotype and functional correlates of disease phenotype in deficiency of adenosine deaminase 2 (DADA2), *Journal of Allergy and Clinical Immunology* (2020), doi: https://doi.org/10.1016/j.jaci.2019.12.908.

during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Deficiency of adenosine deaminase 2



Genotype and functional correlates of disease phenotype in deficiency of adenosine deaminase 2 (DADA2)

Pui Y. Lee MD PhD [1,2*], Erinn S. Kellner MD [3], Yuelong Huang PhD [2], Elissa Furutani MD [4], Zhengping Huang [2,5], Wayne Bainter MMsc [1], Mohammed F. Alosaimi MD [1,6], Kelsey Stafstrom MS [1], Craig D. Platt MD PhD [1], Tali Stauber MD [7], Somech Raz MD PhD [7], Irit Tirosh MD [8], Aaron Weiss DO [9], Michael B. Jordan MD [10,11], Christa Krupski DO MPH [10], Despina Eleftheriou MBBS, MRCPCH, PhD [12], Paul Brogan MBChB, FRCPCH, PhD [12], Ali Sobh MD [13], Zeina Baz MD [14], Gerard Lefranc PhD [15], Carla Irani MD MsCE [16],Sara S. Kilic MD [17], Rasha El-Owaidy MD PhD [18], M.R. Lokeshwar MD DCH [19], Pallavi Pimpale MD [20], Raju Khubchandani MD [20], Eugene P. Chambers MD [21,22], Janet Chou MD [1], Raif S. Geha MD [1], Peter A. Nigrovic MD [1,2+] and Qing Zhou PhD [23+]

1. Division of Immunology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
2. Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
3. Division of Allergy / Immunology, Cincinnati Children's Hospital and University of Cincinnati, Cincinnati, OH, USA
4. Dana Farber and Boston Children's Cancer and Blood Disorders Center, Boston, MA, USA
5. Department of Rheumatology and Immunology, Guangdong Second Provincial General Hospital, Guangzhou, China
6. Department of Pediatrics, King Saud University, Riyadh, Saudi Arabia
7. Primary immunodeficiency clinic, Sheba Medical Center, Jeffrey Modell foundation, Tel Hashomer, Israel
8. Pediatric Rheumatology Service, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel-Hashomer, Israel
9. Department of Pediatrics, Maine Medical Center, Portland, ME, USA
10. Division of Bone Marrow Transplantation and Immune Deficiency, Cincinnati Children's Hospital Medical Center and University of Cincinnati, Cincinnati, OH, USA
11. Division of Immunobiology, Cincinnati Children's Hospital Medical Center and University of Cincinnati, Cincinnati, OH, USA
12. University College London, Great Ormond Street Institute of Child Health, London, United Kingdom
13. Department of Pediatrics, Mansoura University Children's Hospital, Faculty of Medicine, Mansoura University, Mansoura, Egypt
14. Department of Pediatrics, St George Hospital University Medical Center, Beirut, Lebanon
15. Institut de Génétique Humaine, UMR 9002 CNRS-Université de Montpellier, Montpellier, France
16. Internal Medicine & Clinical Immunology Department, Hotel Dieu de France Hospital, Saint Joseph University, Beirut, Lebanon
17. Department of Pediatric Immunology and Rheumatology, Uludag University Medical Faculty, Bursa, Turkey
18. Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University, Cairo, Egypt
19. Department of Pediatrics, Lilavati Hospital and Research Centre, Mumbai, India
20. SRCC Children's Hospital, Mumbai, India
21. Department of Surgery, Vanderbilt University Medical Center, Nashville, Tennessee, USA
22. DADA2 Foundation, Nashville, Tennessee, USA
23. Life Sciences Institute, Zhejiang University, China

+ These authors contributed equally

* **Correspondence to:** Pui Y. Lee, MD PhD,
Boston Children's Hospital,
300 Longwood Ave., Boston, MA 02115
pui.lee@childrens.harvard.edu
Phone: 617-525-1084

Conflict of Interest disclosure statement: The authors declare no conflict of interest relevant to the study.

Funding Sources: This work was supported by the National Institute of Health / National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) K08-AR074562 (P.Y.L.), R01-AR065538, R01-AR073201, R01-AR075906 and P30-AR070253 (P.A.N.), the Fundación Bechara (P.A.N.), the Arbuckle Family Foundation for Arthritis Research (P.A.N.), the Rheumatology Research Foundation Investigator Award (P.Y.L.), Boston Children's Hospital Faculty Career Development Award (P.Y.L), the National Natural Science Foundation of China Grants 31771548 and 81971528 (Q.Z), and the Zhejiang Provincial Natural Science Foundation of China Grant LR19H100001 (Q.Z).

Abstract

Background: Deficiency of adenosine deaminase 2 (DADA2) is a syndrome with pleiotropic manifestations including vasculitis and hematologic compromise. A systematic definition of the relationship between *ADA2* mutations and clinical phenotype remains unavailable.

Objective: We tested whether the impact of *ADA2* mutations on enzyme function correlates with clinical presentation.

Methods: DADA2 patients with severe hematologic manifestations were compared with vasculitis-predominant patients. Enzymatic activity was assessed using expression constructs reflecting all 53 missense, nonsense, insertion and deletion genotypes from 152 patients across the DADA2 spectrum.

Results: We identified DADA2 patients presenting with pure red cell aplasia (PRCA, n = 5) or bone marrow failure syndrome (BMF, n = 10). Most patients did not exhibit features of vasculitis. Recurrent infection, hepatosplenomegaly and gingivitis were common in patients with BMF, of whom half died from infection. Unlike DADA2 patients with vasculitis, patients with PRCA and BMF proved largely refractory to tumor necrosis factor inhibitors. *ADA2* variants associated with vasculitis predominantly reflected missense mutations with at least 3% residual enzymatic activity. By contrast, PRCA and BMF were associated with missense mutations with minimal residual enzyme activity, nonsense variants, and insertions / deletions resulting in complete loss of function.

Conclusion: Functional interrogation of *ADA2* mutations reveals an association of subtotal function loss with vasculitis, typically responsive to TNF blockade, whereas more extensive loss is observed in hematologic disease which may be refractory to treatment. These findings establish a genotype-phenotype spectrum in DADA2.

Clinical Implications: Genotype correlates with clinical phenotype and therapeutic response in DADA2.

Capsule Summary: DADA2 is a monogenic disorder with multi-organ system manifestations. We present a cohort of DADA2 patients with severe hematologic defects and describe novel genotype-phenotype correlations based on functional analysis of 53 *ADA2* mutations.

Keywords: adenosine deaminase 2, DADA2, vasculitis, pure red cell aplasia, bone marrow failure

Abbreviations:

ADA2: adenosine deaminase 2
DADA2: Deficiency of ADA2
BMF: Bone marrow failure
CADD: Combined Annotation Dependent Depletion
CVID: combined variable immunodeficiency
GCSF: granulocyte colony stimulating factor
HSCT: Hematopoietic stem cell transplant
PRCA: Pure red cell aplasia
TNF: Tumor necrosis factor
TNFi: TNF inhibitor

Introduction

Deficiency of adenosine deaminase 2 (DADA2) is a monogenic autoinflammatory disease initially characterized as a cause of stroke and systemic vasculitis in young children [1, 2]. Since its initial description in 2014, the clinical spectrum of this condition has expanded considerably, and variable hematologic and immunologic abnormalities have been described in about half of DADA2 patients [3, 4]. Primary presentations of the disease include pure red cell aplasia (PRCA) that mimics Diamond-Blackfan anemia and bone marrow failure (BMF) with variable cytopenia, even without vasculitis or systemic inflammation [5-7]. The severity of these manifestations can result in transfusion dependency in patients with PRCA or a need for hematopoietic stem transplant (HSCT) in those with BMF [8-11]. Some patients present with humoral immunodeficiency and recurrent infection, further complicating our understanding of DADA2 [12, 13]. How mutations in the same gene can present with different phenotypes is poorly understood.

ADA2 is an extracellular enzyme primarily secreted by monocytes and macrophages [14, 15]. While ADA2 is capable of catalyzing the deaminase reaction that converts adenosine to inosine, its physiologic function is not known. Biallelic mutations in the encoding gene *ADA2* (formerly known as *CECR1*) and very low levels of ADA2 enzymatic activity in the peripheral blood are diagnostic of DADA2 [2]. Missense variants are most common but nonsense mutations, insertions / deletions (indels) and splice site mutations have been described [4].

A systematic analysis comparing *ADA2* mutations associated with different clinical phenotypes is lacking. Previous studies have not been able to establish convincing genotype-phenotype correlations, in part due to a limited number of cases and preferential recruitment of patients with a specific phenotype based on the subspecialty of the investigators. Establishing genotype-phenotype correlations has important diagnostic and therapeutic implications. Whereas tumor necrosis factor inhibitors (TNFi) prevent strokes and improve manifestations of

vasculitis in DADA2 [16], their efficacy for PRCA and BMF is less clear. HSCT may be considered earlier for patients with severe hematologic presentations [9].

Here we report 15 new cases of DADA2 with PRCA or BMF as primary presentation. Based on the genetic findings we observed in these patients, we systematically studied *ADA2* mutations from 152 published cases encompassing the different phenotypes by in silico analysis and functional assay. Our results provide strong evidence for genotype-phenotype correlations in DADA2 with potentially direct clinical relevance.

Methods

Patients: These studies were approved by the Institutional Review Boards at Boston Children's Hospital and Brigham and Women's Hospital. We performed retrospective chart review of 15 patients with DADA2 from 12 families. The patients were enrolled through a world-wide collaboration with approval by the local ethics committees. Research diagnostic testing was performed with written informed consent from the parent or guardian and assent when appropriate. Clinical and laboratory data for the cohort are described in Tables E1-E3 in Online Repository).

Literature search: Please see Supplemental methods in Online Repository for details of literature review and criteria for case selection. Cases selected from each publication and their phenotype are detailed in Table E4 in Online Repository. A complete list of mutations from the selected cases are displayed in Table E5 in Online Repository.

Analysis of *ADA2* mutations: Construction of pcDNA3.1 plasmid for expression of wild-type ADA2 was as described [17]. Site-directed mutagenesis was performed using the NEB Q5 mutagenesis kit (New England Biolabs, Ipswich, MA). The list of mutations and primer pairs used to generate mutant constructs are available in Tables E6 in Online Repository. Mutant

constructs were purified using Purelink Quick Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA) and verified by sequencing. Plasmids were transfected into 293T cells using Fugene 6 (Promega, Madison, WI). Medium was collected after 72 hours and ADA2 activity was quantified using an established spectrophotometric assay that couples the release of ammonia from adenosine with the consumption of NADH [2, 17]. Each mutant was analyzed by three independent experiments and measurements were normalized to the activity of wildtype ADA2 from the same run.

Statistical analysis: The Kruskal-Wallis test was used for comparison of ADA2 activity between multiple mutation types and disease phenotypes. Chi-square was used for comparison of mutation types between clinical phenotypes. All tests were two-sided, and $P < 0.05$ was considered significant. Statistical analyses were performed using Prism 5.0 software (GraphPad Software, La Jolla, CA).

Results

A series of DADA2 patients with primary hematologic defects

We present an international cohort of 15 DADA2 patients from 12 families with PRCA (n = 5) or BMF (n = 10) as their primary presentation. Summarized data for the cohort are displayed in Table 1. Clinical manifestations and laboratory data for each patient are provided in Tables E1 and E2 in Online Repository, respectively. The age of onset for PRCA was very early (median 0.3 years, range 0.1 – 12 years); only 1 patient presented after 6 months of age (Table 1). The age of onset was more variable for the BMF group (median 2.2 years, range 0.1 – 13 years). Patients with PRCA displayed normocytic or microcytic anemia with very low reticulocyte count, consistent with defective erythrocyte production. Most patients with BMF had severe neutropenia and mild anemia, while 2 patients had pancytopenia. Consistent with

previous studies, low immunoglobulin levels (IgG, IgM and/or IgA) were common in patients with DADA2 (Table 1). Cases of severe infection have been described in DADA2-associated BMF [8, 18, 19]. Indeed, recurrent infection was more common in the BMF group (80% vs 20% in PRCA group). These patients experienced a variety of infections, and 5/10 patients ultimately succumbed to sepsis (Table E1 in Online Repository). It is noteworthy that two patients (K-1 and L-1) each had one sibling that died from severe infection before the discovery of DADA2, suggesting that mortality for this phenotype is even higher than estimated here.

Unlike patients from previous large series focused on DADA2 as a monogenic vasculitis [1, 2, 20-22], most DADA2 patients in this PRCA / BMF cohort (12/15, 80%) had no history of vasculitis. Two patients with BMF had cutaneous vasculitis and one patient with PRCA developed sudden-onset squinting and transient hemiparesis with MRI findings compatible with a small ischemic stroke. In the BMF group, almost all patients exhibited hepatosplenomegaly and half experienced severe gingivitis, a feature associated with neutropenia [23] that has not previously been reported in DADA2 (Table E1 in Online Repository). Treatment regimens for these patients include disease modifying anti-rheumatic drugs (DMARDs), biologics, intravenous immunoglobulin, granulocyte colony stimulating factor (GCSF) and HSCT. Unlike the success of TNFi therapy for prevention of stroke and treatment of vasculitis [16], most cases in this cohort did not respond to TNFi (Table E1 in Online Repository). In 10 patients that received TNFi, only one (patient C-1 with PRCA and stroke) showed sustained improvement of hematologic features. Three patients showed improvement of vasculitis and/or systemic inflammation but their cytopenia did not improve. One patient with BMF developed *Pseudomonas aeruginosa* sepsis soon after initiation of TNFi.

Patients in this cohort did not exhibit clinical features of autoimmunity. All patients with PRCA showed negative direct Coomb's test. Four patients in the BMF group were found to have

autoantibodies: 2 with low-titer anti-nuclear antibodies, 1 with anti-neutrophil antibodies, and 1 with non-specific anti-neutrophil cytoplasmic antibodies (Table E3 in Online Repository).

Biallelic mutations in *ADA2* were confirmed in all patients (Table E1 in Online Repository). Nine unique *ADA2* mutations were found in this cohort, and two (F212del and K449Nfs*2) were novel variants (Table 2). To confirm the pathogenicity of these mutations, we expressed these variants in 293T cells and measured ADA2 activity using an established spectrophotometric assay [2, 24]. All mutations from our patient cohort displayed minimal residual ADA2 activity (<2% of wildtype; Table 2). Interestingly, among the 7 previously-described mutations, 6 had been described in patients with severe hematologic manifestations without vasculitis [6, 8, 10, 13, 18, 25, 26]. Moreover, whereas most vasculitis-associated *ADA2* mutations in prior studies were missense variants [4], 6 patients in this cohort had homozygous indel mutations resulting in frameshift and early truncation. All siblings in one family (A-1, A-2 and A-3) exhibited PRCA while another pair of siblings had severe neutropenia (J1 and J2). These observations together raised the possibility of genotype differences among the clinical phenotypes in DADA2.

Genotype comparison of vasculitis and hematologic phenotypes in DADA2

To investigate possible genotype-phenotype correlations, we performed a literature review of published DADA2 cases with vasculitis, PRCA or BMF as the primary presentation. A list of included studies and details of case selection are provided in Methods and Table E4 in Online Repository. We reviewed 186 cases, of which 152 were selected for further investigation (Figure 1A). Details of case selection and exclusion are provided in Supplemental Method in Online Repository. Cases that appeared in multiple publications were analyzed only once and those with other phenotypes or incomplete data on *ADA2* mutations were excluded (Table E4 in Online Repository). Because vasculitis is the most common presentation of DADA2, the

vasculitis group (n = 100) was pooled from 11 major case series from around the world to minimize bias and regional differences. Including the 5 cases in our cohort, we identified 38 cases of DADA2 with PRCA as the primary manifestation. The BMF group consisted of 29 cases including the 10 patients from our cohort. Two patients with PRCA and 5 in the BMF group were described to have features of vasculitis.

One notable demographic difference between groups was age at presentation. In line with the observation in our cohort, DADA2 patients with PRCA presented very early in childhood [median age 0.5 years; interquartile range (IQR) 0.2 – 2.6] while those with vasculitis and BMF generally presented later (vasculitis: median 5.0 years, IQR 1.0 - 10.0 vs. BMF: median 5.0 years, IQR 2.0 - 14.0), including many cases diagnosed in adulthood (Kruskal-Wallis test, $p < 0.001$; Figure E1 in Online Repository).

Among the three groups (167 combined patients), 61 unique *ADA2* mutations were found (Figure 1B). Surprisingly, only two of these mutations were shared by all three groups: H112Q and R169Q. The greatest overlap was found between PRCA and BMF groups, with 7 shared mutations not reported in the vasculitis group. Two mutations were shared by the vasculitis and PRCA groups, while another two were shared by the vasculitis and BMF groups. Plotting *ADA2* mutations according to exon location, mutations associated with all three groups were scattered throughout the gene, without preferential concentration in specific domains (Figure 1C).

When the types of mutation were characterized (counting each allele individually), most mutations associated with vasculitis were missense variants (Figure 1D). Less than 10% of the mutant alleles in the vasculitis group belonged to other categories. In contrast, missense mutations accounted only for 53% of the variants in the PRCA group and 72% in the BMF group, respectively (Chi-square, $p < 0.0001$). Indels comprised the majority of remaining mutations for both groups (38% for PRCA and 16% for BMF; Figure 1D). When cases with

compound heterozygous mutations were excluded, all 63 patients in the vasculitis group had homozygous missense mutations while more variable mutation types were found in the PRCA and BMF groups (Chi-square, $p < 0.0001$).

Using histograms to assess the most common *ADA2* variants in each group, only a few overlapping mutations were found between the groups. R169Q was found in multiple patients in all three groups, while the most common mutation associated with vasculitis, G47R, was not seen in the other groups (Figure 2B). In contrast, G358R was seen in patients with PRCA and BMF, but not in those with vasculitis. R169Q and G358R were the only variants in the BMF group found in more than 2 cases.

Functional analysis of *ADA2* mutations

The abundance of indels in the PRCA group suggests that the more detrimental mutations may be associated with this phenotype. However, missense mutations still accounted for more than 50% of variants. To understand whether functional differences exist among mutations groups, we created expression plasmids for each *ADA2* mutant and transfected them into 293T cells. ADA2 enzymatic activity in the supernatant served as a functional readout for each mutation. Constructs for all 53 missense, nonsense, and indel variants from our patient cohort and published cases (Table E5 in Online Repository) were analyzed using this method. Splicing defects were not evaluated as the sequences for aberrantly-spliced complementary DNA are not available.

Our functional analysis confirmed that all mutations caused a reduction in ADA2 activity (Figure 3A). Not surprisingly, early translational termination caused by nonsense mutations and indels with frameshift completely abrogated ADA2 function. Missense variants, on the other hand, showed a wide spectrum of impact ranging from partial to complete loss of enzyme

activity. Stratification by patient phenotype showed significantly greater residual ADA2 activity for mutations associated with vasculitis compared those associated with PRCA or BMF (Kruskal-Wallis test, $p = 0.0002$; Figure 3B). Examination of different cut-off levels revealed that a residual activity of ≥3% effectively segregated half of mutations associated with vasculitis from the other two groups (Chi-square, $p < 0.0001$; Figure 3C,D). All mutations associated with PRCA or BMF displayed residual activity under this threshold aside for Y353H, which demonstrated 4% residual activity.

To ensure that the statistics were not skewed by the greater number of nonsense and indel variants in the PRCA and BMF groups, we repeated the analysis including only missense mutations. A similar pattern was observed, as missense *ADA2* mutations associated with vasculitis displayed significantly more residual enzymatic activity than those associated with the hematologic phenotypes (Kruskal-Wallis $p < 0.0001$; Figure E2A in Online Repository). We applied several in silico prediction algorithms to assess the pathogenicity of missense mutations associated with the three phenotypes. Consistent with our experimental data, analysis by SIFT [27] predicted that mutations associated with vasculitis would impair gene function significantly less than those associated with PRCA or BMF (Figure E2B in Online Repository). Such difference was not predicted by other algorithms (Polyphen2, MutationTaster and CADD; Figure E2C-F in Online Repository). Taken together, these findings suggest that the more deleterious *ADA2* mutations are associated with severe hematologic phenotypes.

Establishing genotype-phenotype correlations in DADA2

Analysis of individual mutations cannot account for patients with compound heterozygous mutations. To evaluate whether the functional studies can be utilized to predict phenotype using actual mutation configurations from patients, we clustered *ADA2* mutations into

three categories using the 3% residual activity cut-off: type A, hypomorphic missense variants with ≥ 3% residual enzymatic activity compared to wildtype ADA2; type B, missense mutations with minimal (<3%) residual activity, and type C, indels and nonsense mutations with complete absence of enzyme activity. Based on the biallelic mutations identified, each patient was assigned to one of 6 groups (AA, AB, AC, BB, BC, CC) that reflected the predicted functional category of both mutations. For example, a patient with two type A mutations was assigned to group AA, while another patient with compound heterozygous type B and type C mutations was assigned to group BC. This method stratifies biallelic mutations for each patient into groups with a gradient of predicted residual ADA2 activity, where groups AA and CC have the highest and the lowest predicted activity, respectively. Patients with splice-site mutations (n = 7) were excluded from this analysis due to the lack of functional data to evaluate these variants.

For each phenotype, the percentage of patients assigned to each mutation group was plotted. Almost all DADA2 patients with vasculitis had at least one mutation with ≥ 3% residual ADA2 function and therefore were distributed to the AA, AB and AC groups (Figure 4). Most of the remaining patients, the majority of whom were homozygous for R169Q, were assigned to the BB group. By contrast, the majority of PRCA and BMF cases were found in the BB, BC, and CC groups, which have lower predicted residual ADA2 function (Chi-square, $p < 0.0001$). To reflect the actual number of cases in each category, all three groups were plotted together in Figure E3 in Online Repository. Accordingly, the prevalence of BMF and PRCA cases was greater in the genotype categories predicted to have lower residual ADA2 activity. These findings support the existence of genotype-phenotype correlations in DADA2, where missense mutations with greater residual enzymatic function favor the development of vasculitis while more detrimental missense mutations, indels and early-termination mutations causing more extensive disruption of protein function are associated with hematologic manifestations.

Discussion

DADA2 was first described as a form of monogenic vasculitis that mimics polyarteritis nodosa. Case reports and small case series have subsequently established severe hematologic defects as an alternate presentation of DADA2. The 15 new cases with PRCA / BMF described in this study represent the largest series to date for the severe hematologic phenotype of DADA2. We found that patients with PRCA tend to present very early in life and that those with BMF exhibit a high rate of mortality from recurrent infections. Extending the functional analysis of *ADA2* mutations to include the more than 150 published cases in the literature, our work provides new evidence for genotype-phenotype correlations in DADA2. Mutations that are most detrimental to protein function, as measured by residual ADA2 activity, are enriched in patients with severe hematologic involvement.

Genotype-phenotype correlations in DADA2 have been difficult to establish due to incomplete penetrance and variable clinical manifestations in family members with identical mutations [21]. Further, large case series have primarily characterized patients with vasculitis. A recent study comparing 12 patients with vasculitis and 10 with PRCA concluded that mutations in the dimerization domain of ADA2 were associated with vasculitis while those in the catalytic domain aligned with PRCA [11]. However, almost all patients with vasculitis shared the G47R missense mutation. Our analysis of a broader range of variants showed that mutations associated with each phenotype were distributed throughout the gene without preferential location to specific domains.

The physiologic function of ADA2 is not fully elucidated and therefore it remains unclear whether the same mechanism underlies the development of vasculitis and hematologic defects. Based on our stratification of *ADA2* mutations, it is possible that only a small amount of ADA2 is required to maintain normal hematopoiesis, since patients with the most detrimental mutations are most prone to developing PRCA and BMF. It remains unexplained why identical mutations

result in PRCA in some patients and BMF in others. Whether ADA2 is produced by specific hematopoietic progenitor cells or acts differentially on these cells as a soluble factor warrants further investigation. Additional modifier genes and extrinsic factors in the bone marrow environment may also contribute to variable phenotype.

How *ADA2* mutations with residual enzymatic function cause vasculitis remains to be determined. Carmona-Rivera et al. recently found that in the absence of ADA2, adenosine can trigger the formation of neutrophil extracellular traps (NET), which stimulates macrophages to produce TNF-α [28]. While this mechanism may explain the basis of vasculitis and the effectiveness of TNFi therapy, it does not account for the observation that patients with the most detrimental mutations (e.g. nonsense and indels with frameshift) often do not present with vasculitis. It remains to be seen whether the same mechanism applies to the hematologic phenotype of DADA2, which seems less responsive to TNFi. Of the 10 patients that received TNFi in this case series, only one patient showed sustained improvement of PRCA after initiation of etanercept and corticosteroids. Three other patients showed improved fever and/or systemic inflammation without amelioration of baseline cytopenia. Recurrent infections are common in patients with BMF, and the addition of TNFi may further compromise immune defense. Additional studies are needed to address whether TNFi should be used routinely for all DADA2 patients.

Critically, the amount of ADA2 generated by our overexpression system is 20-fold higher than the average plasma ADA2 activity (>250 U/L for supernatant from 293T cells, compared to 12 U/L for healthy adult control plasma) [15]. This overexpression increases the dynamic range of our measurements, enabling us to demonstrate that *ADA2* mutations associated with vasculitis provide greater residual function than mutations associated with PRCA and BMF. In clinical practice, measurement of plasma ADA2 is limited by the inability of current techniques to resolve small differences in residual activity, which in most DADA2 patients therefore appears

uniformly very low. We do not expect that the difference between levels of residual ADA2 function confirmed here in vitro will be observable in clinical samples, as would be required to extrapolate an expected clinical course from measured plasma ADA2 activity.

Although we here stratified each case into a phenotypic category based on primary manifestations, DADA2 phenotypes likely represent continua rather than distinct categories. DADA2 patients with vasculitis can develop anemia and leukopenia as part of their clinical course. Similarly, patients with severe hematologic defects remain susceptible to vasculitis. This potential overlap in phenotypic spectra is best illustrated by the R169Q missense variant, one of the most common pathogenic mutations in DADA2. The R169Q substitution renders minimal residual ADA2 activity (category B, <3%) and is found in all phenotypic categories in our analysis. Supporting this view, a wide spectrum of manifestation including stroke, red cell aplasia and profound cytopenias were reported in a cohort of patients with homozygous R169Q mutations [29]. Inference from genotype is also complicated by high prevalence of compound heterozygosity in DADA2. Therefore, our ability to predict phenotype based on genotype alone remains limited and treatment decisions should be guided by clinical findings.

The spectrum of clinical manifestations in DADA2 extends beyond vasculitis, PRCA and BMF. Common variable immunodeficiency (CVID) has been recently described as a manifestation of the disease [13]. It is unclear whether CVID represents a distinct phenotype in DADA2 because many of these patients also exhibited vasculitis and hematologic defects. The prevalence of hypogammaglobinemia in our cohort is similar to the general estimate for all patients [3]. We suspect that humoral immunodeficiency with low immunoglobulin levels likely represents a common clinical feature of DADA2 regardless of the presenting phenotype. DADA2 can also manifest as autoimmunity (systemic lupus erythematosus and anti-phospholipid syndrome) and lymphoproliferative disease [30, 31]. Patients in our cohort did not exhibit these features and autoantibodies were present only in a few patients. With only a

409 limited number of cases reported, it is difficult to establish genotype correlations for these
410 uncommon DADA2 phenotypes.

411 The broad clinical spectrum of DADA2 and variability in patient presentation are well
412 recognized, but little is known about factors that influence disease phenotype. By characterizing
413 a cohort of patients with PRCA or BMF, our work highlights the severity of hematologic
414 manifestations and their associated morbidity and mortality in DADA2 patients. Systematic
415 comparison of *ADA2* mutations in patients with vasculitis, PRCA and BMF through functional
416 analysis revealed a distinct correlation between mutation pathogenicity and disease phenotype.
417 Further studies are needed to determine differences in the underlying pathophysiology of
418 vasculitis and hematologic defects in DADA2.

Acknowledgements

We thank Drs. M. Hershfield, N. Ganson and S. Kelly for assistance with developing the spectrophotometric assay for quantitation of ADA2 activity, and S. Klayme and G. Debo for technical assistance.

Author contributions:

PYL, PAN and QZ designed the study. PYL, ESK, EF, CDP, TS, SR, IT, AW, MBJ, CK, DE, PB, AS, ZB, GL, CI, SSK, RE, ML, PP, RK, ECC, JC, RG and QZ contributed clinical data. PYL, YH, WB, MFA, and KS performed experiments. PYL, ESK, YH, ZH, PAN and QZ analyzed the data. PYL, PAN and QZ drafted the manuscript and all authors revised the manuscript.

References

1. Navon Elkan P, Pierce SB, Segel R, Walsh T, Barash J, Padeh S, et al. Mutant adenosine deaminase 2 in a polyarteritis nodosa vasculopathy. N Engl J Med 2014; 370:921-31.
2. Zhou Q, Yang D, Ombrello AK, Zavialov AV, Toro C, Stone DL, et al. Early-onset stroke and vasculopathy associated with mutations in ADA2. N Engl J Med 2014; 370:911-20.
3. Lee PY. Vasculopathy, Immunodeficiency, and Bone Marrow Failure: The Intriguing Syndrome Caused by Deficiency of Adenosine Deaminase 2. Front Pediatr 2018; 6:282.
4. Meyts I, Aksentijevich I. Deficiency of Adenosine Deaminase 2 (DADA2): Updates on the Phenotype, Genetics, Pathogenesis, and Treatment. J Clin Immunol 2018; 38:569-78.
5. Ghurye RR, Sundaram K, Smith F, Clark B, Simpson MA, Fairbanks L, et al. Novel ADA2 mutation presenting with neutropenia, lymphopenia and bone marrow failure in patients with deficiency in adenosine deaminase 2 (DADA2). Br J Haematol 2019; 186:e60-e4.
6. Hashem H, Egler R, Dalal J. Refractory Pure Red Cell Aplasia Manifesting as Deficiency of Adenosine Deaminase 2. J Pediatr Hematol Oncol 2017; 39:e293-e6.
7. Michniacki TF, Hannibal M, Ross CW, Frame DG, DuVall AS, Khoriaty R, et al. Hematologic Manifestations of Deficiency of Adenosine Deaminase 2 (DADA2) and Response to Tumor Necrosis Factor Inhibition in DADA2-Associated Bone Marrow Failure. J Clin Immunol 2018; 38:166-73.
8. Ben-Ami T, Revel-Vilk S, Brooks R, Shaag A, Hershfield MS, Kelly SJ, et al. Extending the Clinical Phenotype of Adenosine Deaminase 2 Deficiency. J Pediatr 2016; 177:316-20.
9. Hashem H, Kumar AR, Muller I, Babor F, Bredius R, Dalal J, et al. Hematopoietic stem cell transplantation rescues the hematological, immunological, and vascular phenotype in DADA2. Blood 2017; 130:2682-8.
10. Ulirsch JC, Verboon JM, Kazerounian S, Guo MH, Yuan D, Ludwig LS, et al. The Genetic Landscape of Diamond-Blackfan Anemia. Am J Hum Genet 2018; 103:930-47.
11. Ozen S, Batu ED, Taskiran EZ, Ozkara HA, Unal S, Guleray N, et al. A Monogenic Disease with a Variety of Phenotypes: Deficiency of Adenosine Deaminase 2. J Rheumatol 2019.
12. Arts K, Bergerson JRE, Ombrello AK, Similuk M, Oler AJ, Agharahimi A, et al. Warts and DADA2: a Mere Coincidence? J Clin Immunol 2018; 38:836-43.
13. Schepp J, Proietti M, Frede N, Buchta M, Hubscher K, Rojas Restrepo J, et al. Screening of 181 Patients With Antibody Deficiency for Deficiency of Adenosine Deaminase 2 Sheds New Light on the Disease in Adulthood. Arthritis Rheumatol 2017; 69:1689-700.
14. Hashem H, Kelly SJ, Ganson NJ, Hershfield MS. Deficiency of Adenosine Deaminase 2 (DADA2), an Inherited Cause of Polyarteritis Nodosa and a Mimic of Other Systemic Rheumatologic Disorders. Curr Rheumatol Rep 2017; 19:70.
15. Lee PY, Schulert GS, Canna SW, Huang Y, Sundel J, Li Y, et al. Adenosine deaminase 2 as a biomarker of macrophage activation syndrome in systemic juvenile idiopathic arthritis. Ann Rheum Dis 2019.
16. Ombrello AK, Qin J, Hoffmann PM, Kumar P, Stone D, Jones A, et al. Treatment Strategies for Deficiency of Adenosine Deaminase 2. N Engl J Med 2019; 380:1582-4.
17. Lee PY, Huang Y, Zhou Q, Schnappauf O, Hershfield MS, Li Y, et al. Disrupted N-linked glycosylation as a disease mechanism in deficiency of ADA2. J Allergy Clin Immunol 2018.
18. Cipe FE, Aydogmus C, Serwas NK, Keskindemirci G, Boztug K. Novel Mutation in CECR1 Leads to Deficiency of ADA2 with Associated Neutropenia. J Clin Immunol 2018; 38:273-7.
19. Hsu AP, West RR, Calvo KR, Cuellar-Rodriguez J, Parta M, Kelly SJ, et al. Adenosine deaminase type 2 deficiency masquerading as GATA2 deficiency: Successful hematopoietic stem cell transplantation. J Allergy Clin Immunol 2016; 138:628-30 e2.

20. Caorsi R, Penco F, Grossi A, Insalaco A, Omenetti A, Alessio M, et al. ADA2 deficiency (DADA2) as an unrecognised cause of early onset polyarteritis nodosa and stroke: a multicentre national study. Ann Rheum Dis 2017; 76:1648-56.
21. Nanthapisal S, Murphy C, Omoyinmi E, Hong Y, Standing A, Berg S, et al. Deficiency of Adenosine Deaminase Type 2: A Description of Phenotype and Genotype in Fifteen Cases. Arthritis Rheumatol 2016; 68:2314-22.
22. Rama M, Duflos C, Melki I, Bessis D, Bonhomme A, Martin H, et al. A decision tree for the genetic diagnosis of deficiency of adenosine deaminase 2 (DADA2): a French reference centres experience. Eur J Hum Genet 2018.
23. Kyle RA, Linman JW. Gingivitis and chronic idiopathic neutropenia: report of two cases. Mayo Clin Proc 1970; 45:494-504.
24. Lee PY, Huang Y, Zhou Q, Schnappauf O, Hershfield MS, Li Y, et al. Disrupted N-linked glycosylation as a disease mechanism in deficiency of ADA2. J Allergy Clin Immunol 2018; 142:1363-5 e8.
25. Lamprecht P, Humrich JY, Diebold I, Riemekasten G. Diagnosis of deficiency of adenosine deaminase 2 with early onset polyarteritis nodosa in an adult patient with a novel compound heterozygous CECR1 mutation. Clin Exp Rheumatol 2018; 36 Suppl 111:177.
26. Springer JM, Gierer SA, Jiang H, Kleiner D, Deuitch N, Ombrello AK, et al. Deficiency of Adenosine Deaminase 2 in Adult Siblings: Many Years of a Misdiagnosed Disease With Severe Consequences. Front Immunol 2018; 9:1361.
27. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res 2003; 31:3812-4.
28. Carmona-Rivera C, Khaznadar SS, Shwin KW, Irizarry-Caro JA, O'Neil LJ, Liu Y, et al. Deficiency of adenosine deaminase 2 triggers adenosine-mediated NETosis and TNF production in patients with DADA2. Blood 2019; 134:395-406.
29. Van Montfrans JM, Hartman EA, Braun KP, Hennekam EA, Hak EA, Nederkoorn PJ, et al. Phenotypic variability in patients with ADA2 deficiency due to identical homozygous R169Q mutations. Rheumatology (Oxford) 2016; 55:902-10.
30. Skrabl-Baumgartner A, Plecko B, Schmidt WM, Konig N, Hershfield M, Gruber-Sedlmayr U, et al. Autoimmune phenotype with type I interferon signature in two brothers with ADA2 deficiency carrying a novel CECR1 mutation. Pediatr Rheumatol Online J 2017; 15:67.
31. Trotta L, Martelius T, Siitonen T, Hautala T, Hamalainen S, Juntti H, et al. ADA2 deficiency: Clonal lymphoproliferation in a subset of patients. J Allergy Clin Immunol 2018; 141:1534-7 e8.

Figure Legends

Figure 1. Analysis of *ADA2* mutations by patient phenotype. A) Schematic of literature review and case selection for mutation analysis. B) Venn diagram of unique *ADA2* mutations illustrating overlaps between disease phenotypes. C) Display of *ADA2* gene structure illustrating the distribution of mutations associated with different phenotypes. Shared mutations are displayed by color coding. D) Circle charts illustrating the types of mutations associated with each phenotype. Analysis of individual alleles is displayed in the upper panel while analysis of homozygous individuals is shown in the lower panel.

Figure 2. Analysis of common mutations associated with each disease phenotype. A) Histogram display of allelic count for the most common mutations associated with each disease phenotype. All cases in the current cohort and those selected from literature review were included. B) Phenotype distribution of the most common mutation association with vasculitis (G47R), PRCA (G358R) and BMF (R169Q).

Figure 3. Functional analysis of *ADA2* mutations in vitro. A) ADA2 enzyme activity of individual mutant constructs sorted by mutation type. B) ADA2 enzyme activity of individual mutant constructs sorted by disease phenotype. C) Stratification of mutations within each disease phenotype according to various cut-off values of residual ADA2 enzyme activity. D) Bar graph display of residual enzyme activity for individual mutations associated with each disease phenotype. Dotted line in all panels represent the cut-off value of 3% residual activity. For all panels, results are normalized as percentage of residual activity relative to wildtype (WT) ADA2. Each dot or bar represents the average of results from three independent experiments and error bar represents standard deviation.

536 **Figure 4. Genotype to phenotype analysis using patient mutation configurations.**

537 Distribution of patients with vasculitis, PRCA or BMF phenotype in genotype categories

538 assigned based on *ADA2* mutation type and residual enzymatic activity of missense mutations

539 ($p < 0.0001$, Chi-square test). Bars represent the percentage of patients of the given phenotype.

540

541 **Table 1. Summary of clinical characteristics in DADA2 patients with PRCA or BMF**

	PRCA	BMF
Number of cases	5	10
Median age of onset (year)	0.3	2.2
Sex (% female)	40	50
Anemia (%)	100	80
Lymphopenia (%)	0	40
Neutropenia (%)	0	90
Thrombocytopenia (%)	0	30
Low IgG (%)	20	30
Low IgM (%)	40	60
Low IgA (%)	60	50
Recurrent infection (%)	20	80
Stroke (%)	20	0
Skin vasculitis (%)	0	20
Oral ulcers / Gingivitis (%)	20	70
Hepatosplenomegaly (%)	40	90
Death (%)	20	50

542

543 **Table 2. Characterization of *ADA2* mutations**

Protein	cDNA	n	Phenotype	Type	Domain	ADA2 activity (%WT)	Published phenotype [ref#]
G47W	c.139G>T	1	BMF	missense	Dimerization	0.3 ± 0.5	Vasculitis * [25]
R49Afs*13	c.137dupT	4	PRCA, BMF	frameshift	Dimerization	UD	Hemolytic anemia [8]
F178S	c.533T>C	2	BMF	missense	Catalytic	0.8 ± 0.6	PRCA [8]
F212del	c.634_636 delTTC	1	BMF	deletion	Catalytic	0.8 ± 1.2	-
G321E	c.962G>A	1	PRCA	missense	Catalytic	1.8 ± 1.0	BMF [18]
G358R	c.1072G>A	4	BMF	missense	Catalytic	1.7 ± 0.6	PRCA [6]
K449Nfs*2	c.1346_1347 insTT	1	BMF	frameshift	Catalytic	UD	-
K466Tfs*2	c.1397_1403 AGGCTGAdel	1	BMF	frameshift	Catalytic	UD	PRCA [10]
V458D	c.1373T>A	1	BMF	missense	Catalytic	2.4 ± 0.4	BMF [13] Vasculitis * [26]

544

545 Abbreviations: UD, undetectable.

546 * This mutation was previously described in a patient with compound heterozygous mutations.

Figure 1

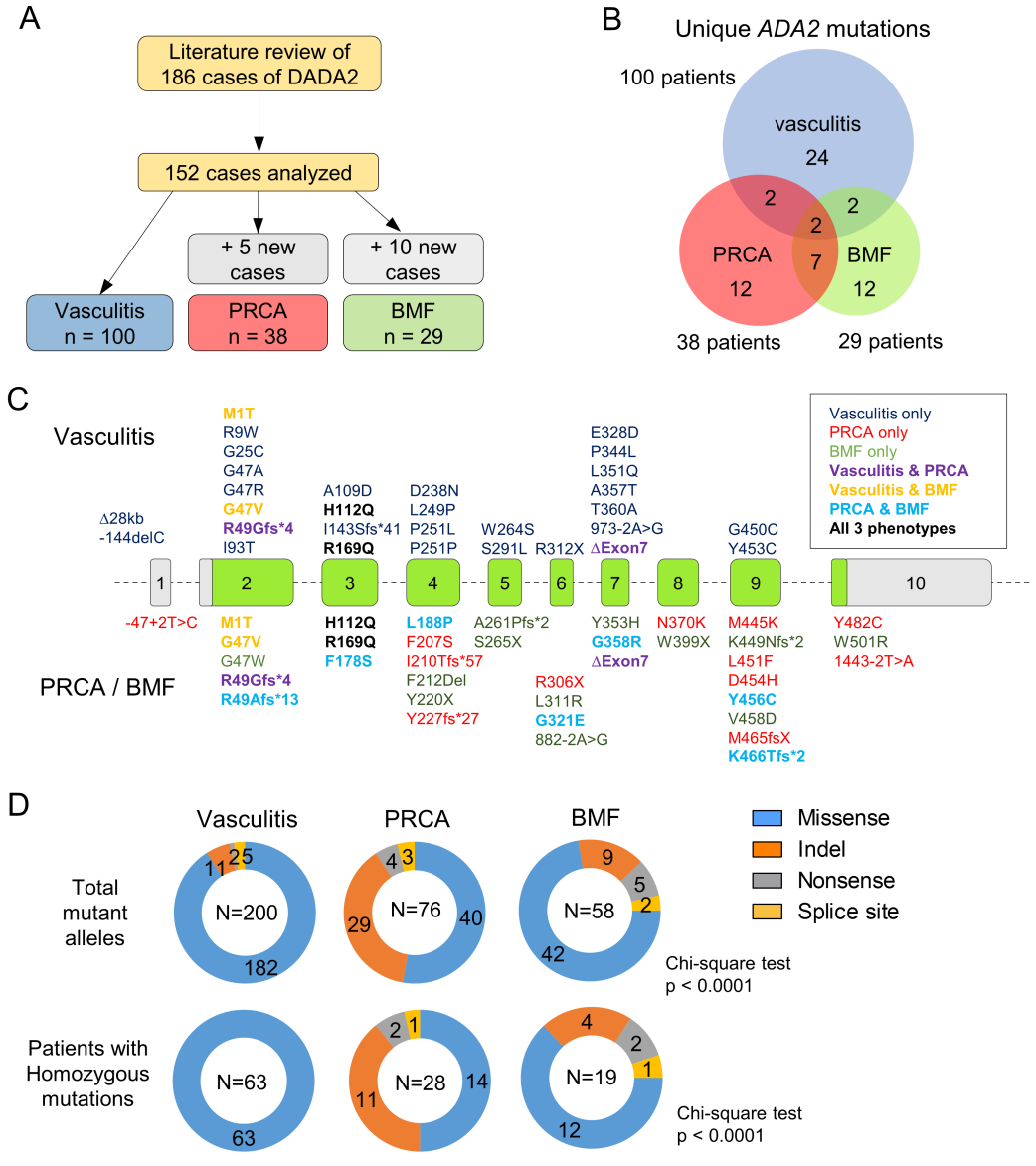

A
Literature review of 186 cases of DADA2
152 cases analyzed
+ 5 new cases
+ 10 new cases
Vasculitis n = 100
PRCA n = 38
BMF n = 29
B
Unique ADA2 mutations
100 patients
vasculitis
24
2
2
2
PRCA
12
7
BMF
12
38 patients
29 patients
C
Vasculitis
PRCA / BMF
Vasculitis only
PRCA only
BMF only
Vasculitis & PRCA
Vasculitis & BMF
PRCA & BMF
All 3 phenotypes
Δ28kb
-144delC
M1T
R9W
G25C
G47A
G47R
G47V
R49Gfs*4
I93T
A109D
H112Q
I143Sfs*41
R169Q
D238N
L249P
P251L
P251P
W264S
S291L
R312X
E328D
P344L
L351Q
A357T
T360A
973-2A>G
ΔExon7
G450C
Y453C
1
2
3
4
5
6
7
8
9
10
-47+2T>C
M1T
G47V
G47W
R49Gfs*4
R49Afs*13
H112Q
R169Q
F178S
L188P
F207S
I210Tfs*57
F212Del
Y220X
Y227fs*27
A261Pfs*2
S265X
R306X
L311R
G321E
882-2A>G
Y353H
G358R
ΔExon7
N370K
W399X
M445K
K449Nfs*2
L451F
D454H
Y456C
V458D
M465fsX
K466Tfs*2
Y482C
W501R
1443-2T>A
D
Vasculitis
PRCA
BMF
Missense
Indel
Nonsense
Splice site
Total mutant alleles
N=200
11
25
182
N=76
4
3
29
40
N=58
9
5
2
42
Chi-square test
p < 0.0001
Patients with Homozygous mutations
N=63
63
N=28
2
1
11
14
N=19
4
2
1
12
Chi-square test
p < 0.0001

Figure 2

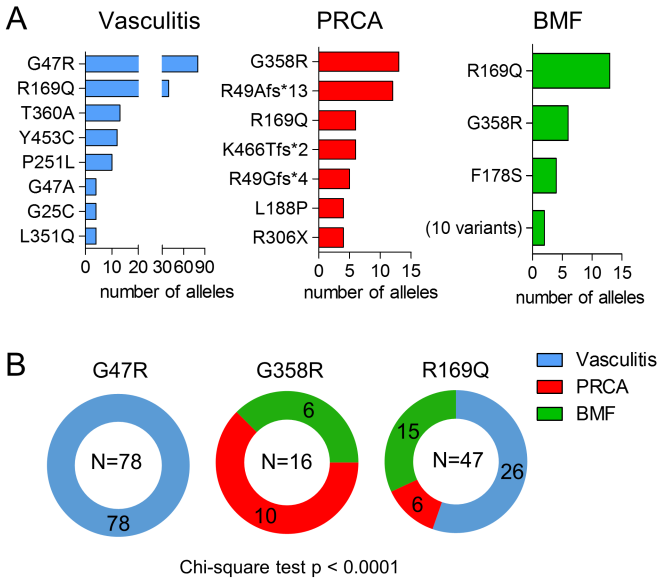
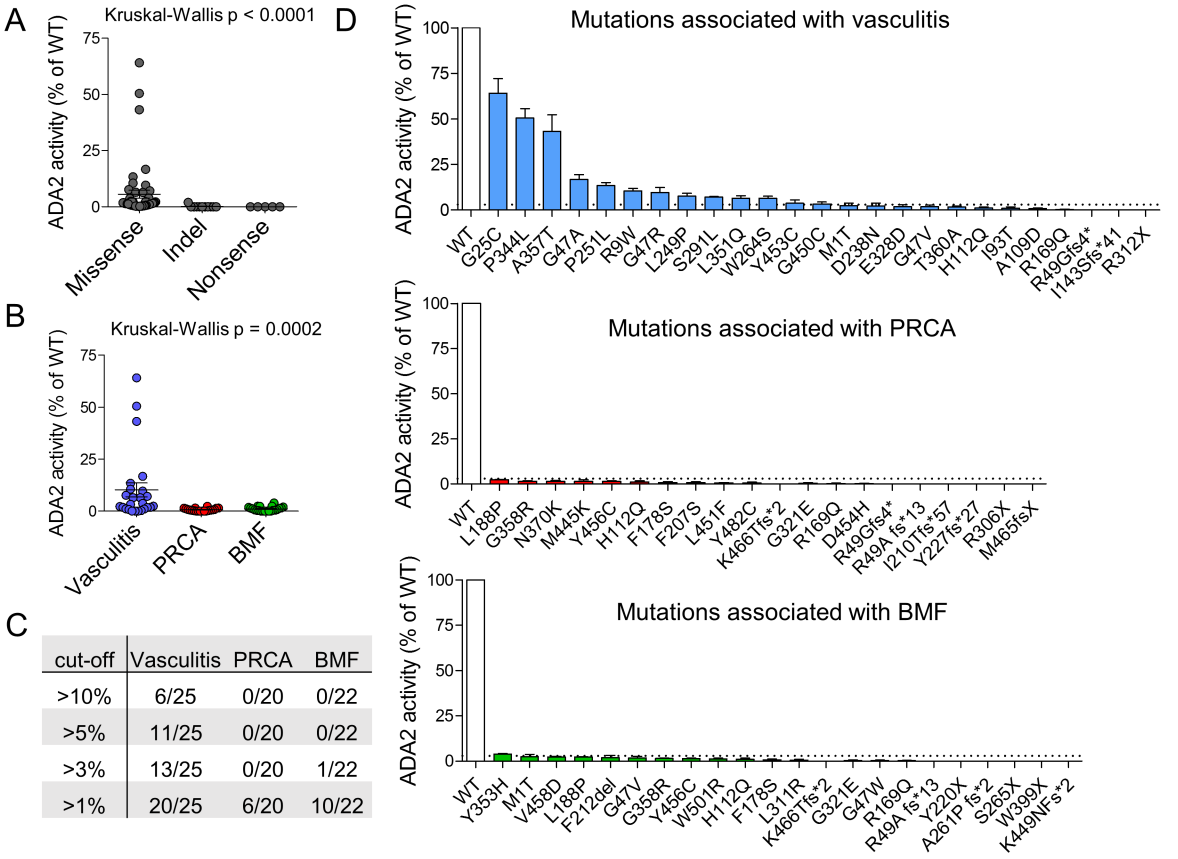


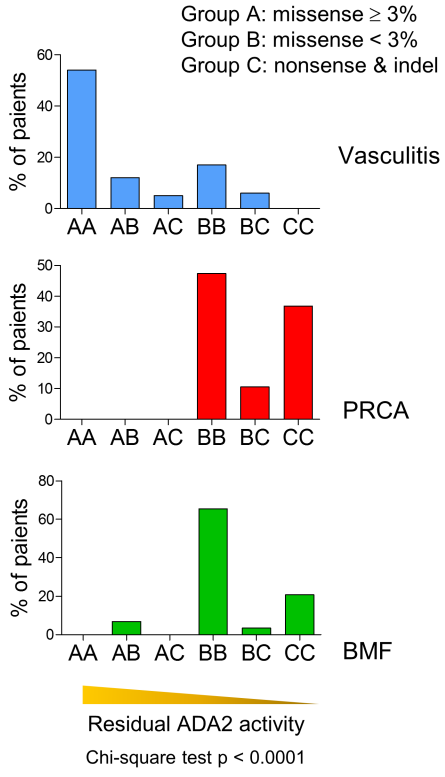
Figure 3

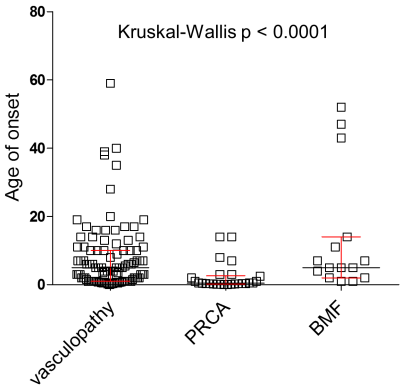


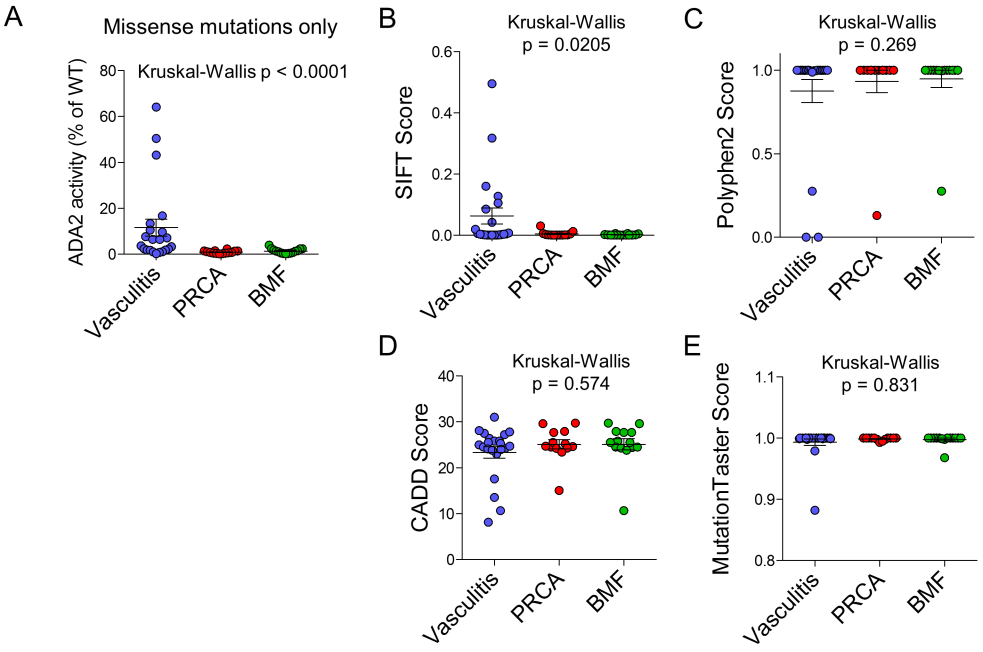
C

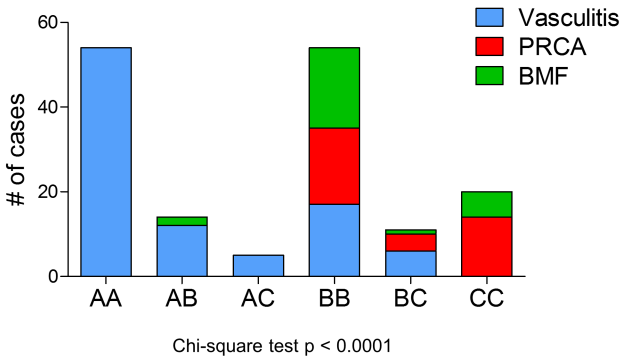
cut-off	Vasculitis	PRCA	BMF
>10%	6/25	0/20	0/22
>5%	11/25	0/20	0/22
>3%	13/25	0/20	1/22
>1%	20/25	6/20	10/22

Figure 4









Genotype and functional correlates of disease phenotype in Deficiency of Adenosine Deaminase 2 (DADA2)

Lee et al.

Online Repository

Supplemental Methods

Literature review and case selection: We performed a comprehensive literature review of DADA2 case series and case reports published between February 2014 to July 2019. The PubMed database was queried using the search terms “DADA2”, “deficiency of adenosine deaminase 2” or “adenosine deaminase 2.” A total of 186 cases were reviewed and assigned to categories of vasculitis, PRBC, BMF or other phenotypes (including lymphoproliferation and asymptomatic patients). Cases selected from each publication and their phenotype are detailed in Table E4. Duplicate cases that appeared in multiple publications were analyzed only once. Cases with other phenotypes or incomplete data on *ADA2* mutations were excluded.

The vasculitis phenotype was defined by any clinical or biopsy-proven diagnosis of polyarteritis nodosa (PAN), cutaneous vasculitis, ischemic stroke, hemorrhagic stroke, or vasculitis of visceral organs. Applying these criteria to 11 major case series from around the world, we identified 100 cases of DADA2 with vasculitis as the predominant phenotype. Because the number of vasculitis cases is disproportionally higher than other phenotypes in existing case series, case reports of patients with vasculitis were not included.

Whereas primary hematologic presentations are less common, we compiled all cases with PRCA and BMF from the literature search. The PRCA group (n = 33) comprised of patients with severe anemia as the presenting features of DADA2, with minimal impact on other cell lineages. The BMF phenotype (n = 19) included cases with initial presentation of severe leukopenia, neutropenia, lymphopenia and/or thrombocytopenia. A complete list of mutations from the selected cases are displayed in Table E5.

Diagnostic testing for DADA2: Biallelic mutations in *ADA2* were confirmed for each patient by DNA sequencing (targeted Sanger sequencing, n = 1; next generation sequencing panels, n= 7; whole exome sequencing, n = 7). Plasma ADA2 enzyme activity was confirmed as low in 7 patients.

Supplemental Figure Legends

Figure E1. Age of symptom onset stratified by disease phenotype. Scatter dot plot display of age of symptom onset for DADA2 patients stratified by disease phenotype. All cases from the current cohort and those selected from literature review were included. Median and interquartile range are displayed.

Figure E2. Functional and in silico analysis of missense *ADA2* mutations. A) Residual ADA2 enzymatic activity of missense mutants grouped by disease phenotype. B-E) Predicted pathogenicity of *ADA2* missense variants by in silico algorithms including B) SIFT, C) Polyphen2, D) MutationTaster and E) Combined Annotation Dependent Depletion (CADD) score.

Figure E3. Distribution of DADA2 cases by genotype categories. Bar graph illustration of DADA2 cases stratified by disease phenotype and genotype category.

Table E1. Clinical characteristics of DADA2 patients with PRCA or BMF

ID	Onset (yr)	Sex	Mutations	Phenotype	Stroke	Skin vasculitis	HSM	GI	Recurrent infection		Treatment	Response to TNFi	Alive
A-1	0.3	M	R49Afs*13 / R49Afs*13	PRCA	No	No	No	-	No	-	transfusion, CS	-	Yes
A-2	0.5	F	R49Afs*13 / R49Afs*13	PRCA	No	No	No	-	No	-	transfusion, AD, tacrolimus	No	Yes
A-3	0.1	F	R49Afs*13 / R49Afs*13	PRCA	No	No	No	-	No	-	transfusion, AD	No	Yes
B-1	12	M	G321E / G321E	PRCA	No	No	Yes	oral ulcer, colitis	No	-	Epo, CS, transfusions, HSCT	-	No
C-1	0.3	M	G358R / G358R	PRCA	Yes	No	Yes	-	No	-	transfusions, CS, ET	Yes	Yes
D-1	0.8	M	F178S / F178S	BMF	No	Yes	Yes	-	No	-	transfusion, IVIG, CS, MMF	-	Yes
E-1	12	F	K466Tfs*2 / K466Tfs*2	BMF	No	No	Yes	-	Yes	pneumonia, anal abscess, sepsis, necrotizing fasciitis	RTX, IVIG, CS, GCSF	-	No
F-1	4	F	R49Afs*13 / R49Afs*13	BMF	No	No	Yes	gingivitis	Yes	pneumonia, URI, UTI	GCSF, AD	No	Yes
G-1	3	M	G47W / G47W	BMF	No	No	Yes	colitis	Yes	necrotizing colitis, sepsis	Anakinra, MMF, sirolimus, AZA, RTX, GCSF, AD	No *	No
H-1	1.3	F	G358R / G358R	BMF	No	Yes	Yes	oral ulcer	Yes	Sinusitis, pneumonia	CS, ET, AD	Partial ±	Yes
I-1	13	M	F212Del / V458D	BMF	No	No	Yes	oral ulcer	Yes	pneumonia, otitis media, sepsis	GCSF, ET, HSCT	No	No
J-1	0.1	F	G358R / G358R	BMF	No	No	Yes	gingivitis	Yes	endocarditis, sepsis	GCSF, CS, AD	No **	No
J-2	0.4	M	G358R / G358R	BMF	No	No	Yes	gingivitis, anal fistula	Yes	sepsis, fungal sinusitis	GCSF, CS, HSCT	-	No
K-1	10	F	F178S F178S	BMF	No	No	No	gingivitis, oral ulcer colitis	No	-	CS, infliximab, methotrexate	Partial ±±	Yes +
L-1	0.4	M	K449Nfs*2 / K449Nfs*2	BMF	No	No	Yes	Oral ulcer, gingivitis	Yes	otitis media, pneumonia, cellulitis	ET, CS, GCSF	Partial ±±	Yes +

Abbreviations: AD, adalimumab; AZA, azathioprine; CS, corticosteroids; ET, etanercept; GCSF, granulocyte colony stimulating factor; Epo, erythropoietin; HSCT, hematopoietic stem cell transplant; HSM, hepatosplenomegaly; IVIG, intravenous immunoglobulins; MMF, mycophenolate mofetil; RTX, rituximab; URI, upper respiratory tract infection; UTI, urinary tract infection; * Patient received two doses of AD prior to death. ** Patient J-1 developed sepsis shortly after initiation of TNF inhibitor. ± Patient H-1 showed improvement of fever and skin rash but cytopenia did not resolve. ±± Patient K-1 and L-1 showed improvement of fever, oral ulcer and gingivitis but cytopenia remained the same. + Patients K-1 and L-1 each has a sibling who died from severe infection.

Table E2. Laboratory findings of DADA2 patients with PRCA or BMF

ID	Phenotype	Hgb (g/dL)	Retic (%)	MCV	WBC (10^9/L)	ANC (10^9/L)	ALC (10^9/L)	PLT (10^9/L)	CD4+ T (10^6/L)	CD8+ T (10^6/L)	CD19+ (10^6/L)	CD56+ (10^6/L)	IgG (mg/dL)	IgM (mg/dL)	IgA (mg/dL)
A-1	PRCA	4.7	0.30	68.7	7.5	825	5.9	481	1746	1587	1029	327	871	28	104
A-2	PRCA	7.1	0.30	83.7	11.8	5.7	5.8	442	1769	1235	1893	196	934	43	13
A-3	PRCA	4.9	0.30	86.7	13.1	3.7	8	337	3006	1142	3107	397	438	30	10
B-1	PRCA	6.8	0.10	91	3.8	2	1.2	252	249	165	42	28	660	178	25
C-1	PRCA	3.3	0.29	62	12.6	2.9	8.8	300	986	427	1127	100	303	73	11
D-1	BMF	6	0.30	90.4	3.2	1	2.1	41	1000	763	84	700	651	44	41
E-1	BMF	10.9	0.10	78.1	1	0.17	0.81	424	219	293	0	6	130	22.6	22.6
F-1	BMF	10.4	2.20	72.5	1.57	0.33	0.7	163	174	424	76	10	735	30	66
G-1	BMF	7.7	0.04	89.6	0.42	0.03	0.37	14	70	170	0	0	1330	33	70
H-1	BMF	12.1	0.8	74	2.1	1.26	0.4	114	336	59	28	112	487	12	28
I-1	BMF	11.6	1.50	75.8	2.01	0.24	1.38	180	365	262	31	64	274	6	33
J-1	BMF	8	2.00	75	5	0.05	5	250	2512	1507	2679	586	2040	767	69
J-2	BMF	9.8	2.40	69	5	0.05	4	600	1980	1584	220	484	1130	24	68
K-1	BMF	6.9	1.50	65	2	0.3	3	250	621	621	336	251	1010	89	163
L-1	BMF	9.5	2.60	75	7	0.3	6.5	250	735	8188	315	2730	1290	76	365

Abbreviations: Hgb, hemoglobin; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; MCV, mean corpuscular volume; Retic, reticulocyte.

Table E3. Autoantibody profiles in DADA2 patients with PRCA or BMF

ID	Phenotype	DAT	ANA	ANCA	Anti-dsDNA	Anti-ENA	Anti-cardiolipin	Anti--β2GP	Anti-neutrophil	Anti-platelet
A-1	PRCA	Neg								
A-2	PRCA	Neg								
A-3	PRCA	Neg								
B-1	PRCA	Neg	Neg				Neg			
C-1	PRCA	Neg	Neg							
D-1	BMF	Neg								
E-1	BMF		+ 1:100		Neg					
F-1	BMF		Neg	+ c-ANCA*	Neg					
G-1	BMF		Neg	Neg		Neg			Neg	
H-1	BMF		Neg				Neg	Neg	Neg	
I-1	BMF	Neg			Neg				Neg	Neg
J-1	BMF	Neg	Neg	Neg	Neg				Neg	
J-2	BMF									
K-1	BMF	Neg	Neg				Neg	Neg		
L-1	BMF	Neg	+ 1:160						+	

* Proteinase-3 and myeloperoxidase specific antibody testing were negative

Abbreviations: ANA: anti-nuclear antibodies; ANCA: anti-neutrophil cytoplasmic antibodies; β2GP: β2 glycoprotein; DAT: direct antiglobulin test; dsDNA: double-stranded DNA; ENA: extractable nuclear antigens; Neg: negative.

Table E4. Selection of cases from literature review

Authors	PMID#	Total	Vasculitis	PRCA	BMF	Excluded (reason*)	
Alsultan et al.	29271561	1	0	0	1	0	
Barzaghi et al.	30692987	1	0	0	1	0	
Batu et al.	26233953	6	3	0	0	3	3 (duplicate)
Ben-Ami et al.	27514238	5	0	4	1	0	
Caorsi et al.	28522451	15	14	0	0	1	1 (other phenotype)
Cipe et al.	29564582	1	0	0	1	0	
Claassen et al.	30559313	1	0	1	0	0	
Ghurye et al.	30924144	2	0	0	2	0	
Gibson et al.	31008556	9	8	0	0	1	1 (partial genotype)
Hashem et al.	28974505	14	0	3	5	6	6 (duplicate)
Hashem et al.	28230570	1	0	1	0	0	
Hsu et al.	27130863	1	0	0	1	0	
Michniacki et al.	29411230	2	0	0	2	0	
Nanthapisal et al.	27059682	15	10	0	0	5	5 (other phenotype)
Navon-Elkan et al.	24552285	24	20	0	0	4	4 (2-partial genotype; 2-other phenotype)
Neishabury et al.	31097629	2	0	2	0	0	
Ozen et al.	31043544	24	6	10	0	8	8 (2-partial genotype ; 6-duplicate)
Rama et al.	29681619	13	13	0	0	0	
Sahin et al.	28516235	8	6	0	0	2	2 (Duplicate)
Sasa et al.	n/a *	2	0	2	0	0	
Sundin et al.	29620681	1	0	0	1	0	
Trotta et al.	29391253	9	3	0	3	3	3 (other phenotype)
Ulirsch et al.	30503522	9	0	9	0	0	
Van Eyck et al.	25457153	2	0	0	1	1	1 (other phenotype)
Von Montfrans et al.	26867732	9	8	1	0	0	
Zhou et al.	24552284	9	9	0	0	0	
Total cases reviewed		186	100	33	19	34	
Lee et al. (current)		15	0	5	10		
All cases combined		**201**	**100**	**38**	**29**		

* Duplicated cases were each analyzed only once. Partial genotype refers to patients with only one identified mutation. Other phenotypes include patients without frank features of vasculitis or hematologic abnormalities, asymptomatic individuals, and patients with primarily lymphoproliferative disease without features of other phenotypes.

** Pubmed ID not available. Abstract reference: Sasa et al. *Blood* 2015 126:3615

Table E5. *ADA2* mutations grouped by patient phenotype.

	Vasculitis			PRCA		BMF	
missense	M1T	H112Q	P344L	G47W	G358R	M1T	G321E
	R9W	R169Q	L351Q	H112Q	N370K	G47V	Y353H
	G25C	D238N	A357T	R169Q	M445K	H112Q	G358R
	G47A	L249P	T360A	F178S	L451F	R169Q	Y456C
	G47R	P251L	G450C	L188P	D454H	F178S	V458D
	G47V	W264S	Y453C	F207S	Y456C	L188P	W501R
	I93T	S291L		G321E	Y482C	L311R	
	A109D	E328D					
non-sense	R312X			R306X		Y220X S265X W399X	
Indels	R49Gfs4* I143Sfs*41			R49Gfs4* R49Afs*13 I210Tfs*57 Y227fs*27 M465fsX K466Tfs*2		R49Afs*13 F212del A261Pfs*2 K449NFs*2 K466Tfs*2	
Splicing	c.753G>A c.973-2A>G			c.47+2T>C c.1443-2T>A		c.882-2A>G	
Other	exon7 deletion 28kb large deletion -144delC promoter deletion			exon7 deletion			

Table E6. Primer sequences for site directed mutagenesis of ADA2 construct

Mutation	Forward Primer	Reverse Primer
M1T	gaattcaccacgttggtggatg	tgcagatatccagcacag
R9W	cccatctgagtggccagccct	ccatccaccaacatggtgaattctg
G25C	gtctttcttctgctcagctctatcc	attgccacagccaacagc
G47A	gatgcggctggcggggcggctg	atcttttctttcaacaacagatgcgcccgtg
G47R	ctgagggggcggctggtgctgaa	ccgcatcatctttttctttcaacaacagatgcgccc
G47V	gatgcggctggtggggcggctg	atcttttctttcaacaacagatgcgcccgtg
G47W	gatgcggctgtgggggcggctg	atcttttctttcaacaacagatgcgcccgtg
R49Gfs4*	gctgggggggggctggtgctg	cgcatcatctttttctttcaacaacagatgc
R49A fs*13	cggctgggggcgcggctggtg	catcatctttttctttcaacaacagatgcgccc
I93T	aagcatctcactgagagaagtc	ggcctggaaaaagtgcat
A109D	cttgcacctccatgacattgg	gcatccccttttggcatcatcc
H112Q	ggctgccttgcagctccatgaca	ccttttggcatcatccttagaatattaaacacttg
I143Sfs*41	tcatgcagttcagatttgctcac	ccccccttggggtgaaaca
R169Q	ggaggattatcagaagcgggtgc	agcagaatccacttggaac
F178S	gtcactgagtctgatgacagcttg	gttctgcacccgcttccg
L188P	gaatttcactccggtgacccagc	ctcagcaagctgtcatcaaac
F207S	ctggtcgaaatctgaaaccatct	acaacattttggtttgtgtaaatc
I210Tfs*57	cttcttcaccatctctgg	tggtttcaaatttcgacc
F212del	accatctctggtctcatc	gaagatggtttcaaatttcg
Y220X	agaagaggatctgtgagcaccagtgttcagagac	gagatgagtttttgttcatggatgagaccagagatg
Y227fs*27	tgtcttccggagcatgca	gtctctgaacactggtgc
D238N	gttctacgagaacaacgtgctctac	tcctgcatgctccggaag
L249P	agagccaggcctctgccggtgt	gatctccatgtagagcacgttg
P251L	caggctgctgctggtgtatgagct	gctctgatctccatgtagagcacg
D261P fs*2	agagcaccatccataacgaagagtgg	ccactgagctcatacacc
W264S	tgacgaagagtcgtcagtgaagac	tggtgctctccactgagc
S265X	agaagaggatctgtgagtgaagacttaccaggaag	gagatgagtttttgttcccactcttcgtcatggtg
S291L	aatcatttatttggatcacagatc	ttgattccaataaactcagg
R306X	agaagaggatctgtgaatggccatggggctccga	gagatgagtttttgttcgatggattctgcgatgacagcc
L311R	gccatggggcgccgaatcaagt	cattcggatggattctgcgatg
R312X	agaagaggatctgtgaatcaagttccccacggtg	gagatgagtttttgttcgagccccatggccattcg
G321E	ggtggtggcagagtttgacctg	gtggggaacttgattcgg
E328D	ggtggggcatgacgacactggcc	aggtcaaaccctgccacc
P344L	tctgatgatcctcgccaaggatg	gcttccttgtagtcatgcaag
L351Q	ggcgttaagcagccttacttc	atccttggcggggatcat
Y353H	taagctgcctcacttcttccacgcc	acgccatccttggcgggg
A357T	cttcttccacaccggagaaacag	taaggcagcttaacgcca
G358R	tcttccacgcccgagaaacagac	agtaaggcagcttaacgc
T360A	cgccggagaagcagactggca	tggaagaagtaaggcagcttaac
N370K	catagacaggaagattctggatgc	gaagtaccctgccagtct
W399X	agaagaggatctgtgaaaaaaggacatccccatag	gagatgagtttttgttcggagtaagtcctgactgc
M445K	tgacccagctaagtttggtgcca	tcagagctgatcaccatgg
G450C	tggtgccaaatgcttgtcctatg	aacatagctgggtcatcag
K449Nfs*2	ttaggcttgtcctatgatttc	ttggcaccaaacatagctg
L451F	gccaaaggcttttcctatgatttc	accaaacatagctgggtc
D454H	cttgtcctatcatttctatgaggtc	cctttggcaccaaacatag
Y453C	aggcttgtcctgtgatttctatg	ttggcaccaaacatagctg
Y456C	ctatgatttctgtgaggtcttcatgg	gacaagcctttggcacca
V458D	gatttctatgaggacttcatgggc	ataggacaagcctttggc
M465fsX	agaagaggatctgtgaaaggctgacctgaggacc	gagatgagtttttgttccccccaatgcccatgaa
K466Tfs*2	ctgaggaccctcaaacagc	gtcatccccccaatgccca
Y482C	ctctatcaagtgcagtaccctgttg	ttcatggccagctgtttg
W501R	gaagaagagacgggataagttcatag	cagatttccatgaaagtatttttc