Diagnostic capabilities, clinical features, and longitudinal UBA1 clonal dynamics of a nationwide VEXAS cohort

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Data availability statement

All data and results of the survey from which results were generated are available in the manuscript. Additional request can be directed via email to the corresponding author.

Consent for publication

Informed consent obtained according to the protocols approved by the Institutional Review Board of the participating institutions and in accordance with the ethical principles set forth by the Declaration of Helsinki.

Abstract

VEXAS is a prototypic hemato-inflammatory disease combining rheumatologic and hematologic disorders in a molecularly defined nosological entity. In this nationwide study, we aimed at screenshotting the current diagnostic capabilities and clinical-genomic features of VEXAS, and tracked *UBA1* longitudinal clonal dynamics upon different therapeutics, including allogeneic hematopoietic cell transplant.

We leveraged a collaboration between the Italian Society of Experimental Hematology and of Rheumatology and disseminated a national survey to collect clinical and molecular patient information.

Overall, 13/29 centers performed *UBA1* genomic testing locally, including Sanger sequencing (46%), next-generation sequencing (23%), Droplet Digital PCR (8%), or combination (23%). A total of 41 male patients were identified, majority (51%) with threonine substitutions at Met41 hotspot, followed by valine and leucine (27% and 8%). Median age at VEXAS diagnosis was 67 years. All patients displayed anemia (median hemoglobin 9,1 gr/dL), with macrocytosis. Bone marrow vacuoles were observed in most cases (89%). The most common rheumatologic association was polychondritis (49%). A concomitant MDS was diagnosed in 71% of patients (n=28), chiefly exhibiting lower IPSS-R risk profiles. Karyotype was normal in all patients, except 3 MDS cases showing -Y, t(12;16)(q13;q24), and +8. The most frequently mutated gene was *DNMT3A* (n=10), followed by *TET2* (n=3). At last follow-up, 5 patients died and 2 patients progressed to acute leukemia. Longitudinal *UBA1* clonal dynamics demonstrated mutational clearance following transplant.

We collected a nationwide interdisciplinary VEXAS patient cohort, characterized by heterogeneous rheumatologic manifestations and treatments used. MDS was diagnosed in 71% of cases. Patients exhibited various longitudinal *UBA1* clonal dynamics.

Abbreviations

Allo-HCT

AML: acute myeloid leukemia

ARCH: Age-related clonal hematopoiesis

BM bone marrow

CHIP: Clonal hematopoiesis of indeterminate potential

ddPCR: droplet digital polymerase chain reaction

IPSS-R/M: Revised/Molecular International Prognostic Scoring System

MDS: myelodysplastic neoplasm/syndromeVPa

MGUS: Monoclonal Gammopathy of Undetermined Significance

NGS: next generation sequencing

SIES: Italian Society of Experimental Hematology

SIR: Italian Society of Rheumatology

VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory and somatic)

WES: whole exome sequencing

Introduction

VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory and somatic) syndrome is a prototypic hemato-inflammatory disease juxtaposing for the first time rheumatologic and hematologic disorders under the aegis of a molecularly defined nosological entity.(1) Given its multifaceted clinical presentations, VEXAS represents a challenging masquerade syndrome and it has elicited a wide medical interest. What makes this condition so hard

to be diagnosed is the variety of clinical manifestations that, taken individually, can be ascribed to many other disorders. Indeed, VEXAS lies at the interface between bone marrow failure syndromes (macrocytic anemia), "inflamm-aging" and clonal hematopoiesis, displaying a tetrad of somatic mutations, morphologic bone marrow (BM) features, inflammatory pathways and immune overshooting.(2, 3)

Somatic mutations in the X-linked gene *UBA1* in hematopoietic stem and myeloid cells represent the genetic underpinnings of the syndrome.(4) The presence of *UBA1*-mutant clonal hematopoiesis, typified by the characteristics BM vacuoles(5), and the resultant perturbation of ubiquitylation and hyperinflammation(6), offers a fertile soil for acquisition of further somatic myeloid gene mutations, in a disease continuum which often culminates in the association with myelodysplastic syndromes/neoplasms (MDS).(7) Besides MDS, macrocytic anemia (regardless of an overt MDS diagnosis), plasma cell dyscrasia, and recurrent thrombosis are among the most frequent hematological features, whereas inflammatory manifestations encompass skin (chiefly, Sweet syndrome or Sweet syndrome-like lesions), joints, lung, gastrointestinal, ocular and kidney involvement as well as non-infectious fever, nose and ear chondritis.(8, 9) As a result, collaboration among different specialties is of paramount importance to promptly identify and correctly diagnose patients, usually men in their 6-7th decade of life.(10)

In a study exploring the prevalence of *UBA1* variants in the general population, exome data from 163,096 participants within the Geisinger MyCode Community Health Initiative were queried.(11) Disease-causing *UBA1* variants were found in 1 in ~4000 men over 50 years, and in about 1 in 14,000 individuals, a prevalence similar to that of MDS.(12)

Treatment strategies vary according to the specific clinical presentations and aim at suppressing the condition of hypercytokynemia (e.g., with anti-interleukins), the *UBA1*-mutant clone (azacitidine, allogeneic hematopoietic cell transplant, allo-HCT) or support therapy (growth factors for anemia, thrombotic and infectious prophylaxis, among others).(8, 13, 14)

With the joint efforts of the Italian Society of Experimental Hematology (SIES) and the Italian Society of Rheumatology (SIR), we aimed at screenshoting the current *UBA1* diagnostic capabilities and clinical-genomic features of VEXAS in Italy, 2.5 years since its initial discovery.(1) We also collected patient outcomes and

performed longitudinal molecular studies in cases undergoing different therapeutic approaches, including allo-HCT.

Methods

Patients

This study was conducted through a national survey, under the purview of SIES and SIR, mostly involved in the clinical management of VEXAS patients. An electronic link was disseminated via email from September 2022 to April 2023 with multiple rounds of reminders to ensure wide patient enrollment. Selection included all cases with a diagnosis of VEXAS made by concomitant clinical evaluation and molecular *UBA1* screening (see below). Centers were queried for relevant data concerning diagnostic capabilities (type of molecular analysis, number of tests), and patients' characteristics including main laboratory values and treatment types. Along with this, longitudinal sampling was performed for a sub-cohort of cases with canonical Met41 substitutions to study variations of clonal burden upon different therapeutics. The study was approved by the institutional review board of the participating centers and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Genomic studies

Mutations of the *UBA1* gene were identified in DNA extracted both from peripheral blood (PB) and BM. The fraction of mononuclear cells was isolated by separation with Ficoll-Hypaque. Genomic DNA was extracted using the commercially available QIAamp DNA Blood Mini Kit Qiagen® (Qiagen Srl, Milan, Italy) according to the manufacturer's instructions. *UBA1* mutations were screened by a variety of techniques (Sanger(5), next-generations sequencing-NGS, droplet digital PCR-ddPCR(15), or whole exome sequencing-WES) depending on center availability. To explore the variation of clonal dynamics from diagnosis and during follow-up in a subset of patients with available DNA sampling, we designed ddPCR primers for the 3 hotpots substitutions at Met41 (Thr, Leu, Val) as previously shown for the Met41Val substitution.(15) In addition, a 30-genes myeloid NGS panel (SOPHiA GENETICS, Saint-Sulpice, Switzerland) was used to identify concomitant mutations on a MiniSeq®

sequencing platform (Illumina, San Diego, California) for 23/41 cases with a strong suspicion for concomitant MDS, and calculate appropriate disease risk scoring systems, as detailed elsewhere.(16)

Statistics

Patients' characteristics were summarized by descriptive statistics to provide a summary of the collected information on the characteristics of the cohort and distribution of values. Quantitative variables were expressed as median, interquartile range, minimum and maximum. Qualitative variables were expressed as numbers and percentages. Overall survival (OS) was calculated from VEXAS symptoms onset to last follow-up or death and estimated with the Kaplan-Meier method. Analyses, graphs and data visualization were generated using the computing environment of Excel Microsoft Office 365, GraphPad Prism (8.4.0) and R software (4.0.0 R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Results

UBA1 genomic testing in Italy: methods, availabilities and alterations

Overall, 41 patients were diagnosed with VEXAS in 29 participating centers, 13 of which had *UBA1* genomic testing available (**Figure 1A**). This consisted of either Sanger sequencing (n=6, 46%), NGS (n=3, 23%) or ddPCR (n=1, 8%), whereas a combination of these methods was in use in 3 (23%) centers (**Figure 1B**). Over a median time of 13,7 months (interquartile range, IQR 4.2-18.5) since *UBA1* testing availability, the positivity rate reached 11,7% (n=41 out of 351 total tests). Approximately half (51%) of VEXAS cases harbored threonine substitutions at the Met41 hotspot, followed by valine and leucine (27% and 8%; **Figure S1**). Of note is that 3 patients had rare mutations of the *UBA1* gene, the new variant c.118-1G>C, identified at the splice acceptor site of exon 3 (n=2) and 1 had the rare c.167C>T, p.Ser56Phe. Furthermore, WES detected in 1 patient the c.1430G>C mutation of the gene *UBA1* in exon 14, which has been recently described and functionally characterized as pathogenic.(17) WES has been performed in this particular case for which the clinical suspicion of VEXAS was very high and the most common exon 3 variants had been excluded by Sanger sequencing.

Patient characteristics

A total of 41 patients, all males and with rheumatologic manifestations were accrued. Median age at VEXAS diagnosis was 67 years (IQR=62-72). All patients had anemia (median hemoglobin 9,1 gr/dL, IQR=8,3-10,8) and macrocytosis (median MCV 105 fL, IQR=97,3-109). The demographic and clinical characteristics of patients with VEXAS syndrome are summarized in **Table 1**. BM vacuoles were observed in myeloid and erythroid precursors, or both in the majority of cases (89%; **Figure 1C**). Skin manifestations were present in roughly a third (34%) of patients with forms ranging from Sweet Syndrome or neutrophilic dermatosis to erythematous plaques and skin rashes, as previously reported.(9) Rheumatological manifestations were heterogeneous, but the most frequent was relapsing polychondritis, typically involving ear and nose (49%, **Figure 1D**).

Notably, 37% of patients also showed MGUS (Monoclonal Gammopathy of Undetermined Significance) at serum protein electrophoresis evaluation, more frequently of the IgG subtype (n=9 IgG, n=3 IgM, none with IgA, n=3 cases with missing data). None of these patients progressed to multiple myeloma at last follow-up. Only in 1 case, a monoclonal B-cell lymphocytosis was associated with VEXAS syndrome.

Finally, 11 patients (27%) experienced thrombotic events, chiefly deep vein thrombosis (DVT), at disease onset which constituted reasons to seek medical attention along with the accompanying macrocytic anemia, and 3 presented more than 1 episode. All but one case (central retinal artery occlusion) originated in the venous district and anatomic sites were various: upper extremity DVT (31%), lower extremity DVT (38%), lower extremity DVT with pulmonary embolism (15%), splanchnic (8%) and superficial vein thrombosis (8%).

The clinical dyad of myelodysplastic syndrome and VEXAS

MDS was diagnosed in 71% (n=28) of our patients (**Figure 2A**), all falling into lower Revised International Prognostic Scoring System (IPSS-R scores <3,5(18)) categories except for two cases, who were still classified as intermediate (≤ 4,5). When re-stratified according to the recently released Molecular International Prognostic Scoring System (IPSS-M)(19), only two cases were upgraded from the IPSS-R moderate to the IPSS-M high-risk group, while the majority remained in the lower-risk categories (negative values, **Figure 2B**). Cytogenetics was normal in all but 3 MDS cases showing chromosome anomalies such as -Y, t(12;16)(q13;q24), and trisomy

8. A total of 2 patients did not satisfy the criteria for MDS but rather belonged to the nosological entity of clonal cytopenia of undetermined significance (CCUS).

Overall, NGS analysis was performed in 23 cases (2 CCUS and 21 VEXAS/MDS), 74% of which harbored at least 1 myeloid gene mutation, with a high frequency of *DNMT3A* (n=10), followed by *TET2* mutations (n=3) with only 22% of these patients having >1 alteration. We previously described the first patient with VEXAS with *ASXL1/SF3B1*-mutant MDS who evolved to AML (acute myeloid leukemia) upon acquisition of a *RUNX1* mutation. We have now recorded an additional case of *TET2*-mutant MDS evolving to AML and succumbing because of disease progression.

Treatment approaches

At the time of diagnosis, all but 3 patients had already received rheumatologic treatments: 12 patients had been treated with corticosteroids only; 20 patients had received during their clinical history at least one immunosuppressive agent, such as cyclosporine A, methotrexate, hydroxychloroquine, colchicine or azathioprine; 11 patients had received anti-interleukin (IL)-1 inhibitor (anakinra and canakinumab); 8 had been treated with anti-IL6 drug (tocilizumab), and 7 with JAK-inhibitors (baricitinib, ruxolitinib, tofacitinib, upadacitinib and filgotinib). Overall, 21 patients underwent more than one line of treatment (including steroids), and the median number of lines of treatment received was 3 (IQR=2-4) in the whole cohort (**Table 1**).

Of the 11 patients experiencing thrombosis, 2 were treated with warfarin, 1 with fondaparinux, 1 initially with fondaparinux and then switched to the new direct oral anticoagulants (DOACs), 4 only with DOACs, and 3 with low-molecular-weight heparins (LMWHs). Overall, 6 patients had discontinued anticoagulant therapy at last follow-up.

The therapeutic approach for patients with MDS associated with VEXAS syndrome was based on disease risk assessment according to general MDS treatment guidelines(20), mostly with erythropoietin and hypomethylating agents (HMA). Of the patients who received erythropoietin treatment, 9 did not show hemoglobin improvement, and 4 achieved a major response with transfusion independency.(21) Two patients received HMA with azacitidine as a first line of treatment (n=1 died because of spontaneous bowel perforation and 1 was switched

to ruxolinitib due to inflammatory flare),whereas 1 patient received it as a second line. Remarkably, of the 2 patients with low-risk MDS progressing to AML, one (carrying the Met41Val genotype) evolved after one year, while the other (Met41Thr) after two years since VEXAS onset.

Three patients underwent allogeneic allo-HCT from a MUD, preceded by reduced intensity conditioning (RIC) based on treosulfan plus fludarabine (Treo/Flu). **Table S1** summarizes the clinical characteristics of these patients. Transplant-related infective complications were registered in 2 cases: 1 patient had a sepsis sustained by Enterococcus faecium, while the other experienced an invasive candidemia. Notably, no patient developed Graft versus Host Disease (GvHD), and all 3 patients are alive and in clinical remission, 4 months, and 10 months (n=2) following transplant.

With a median follow-up of 7.5 months (0.8-13.7), OS of the entire cohort was 95% at 1 year from VEXAS symptoms onset (**Figure 2C**). At last follow-up, 5 patients (all with the Met41Thr genotype, the most common in our cohort) died: 3 of infectious complications, 1 for spontaneous bowel perforation, and 1 for disease progression to AML.

UBA1 **clonal dynamics upon different therapeutics**

We quantified variations of the variant allele fraction (VAF) during the treatment of 4 patients, as shown in **Figure 3**. UPN1 had VEXAS with MDS/ myeloproliferative neoplasm (MDS/MPN) overlap syndrome and underwent ruxolitinib treatment followed by allo-HCT. The PB sample analyzed at disease onset had a VAF of 46,6% which increased to 58,1% after one month of treatment with ruxolitinib, and reached 85,7% at the pre-transplant control. The post-transplant studies showed a near complete abatement of *UBA1* mutation already at +30 days after transplantation in both BM and PB samples (VAF 0,01% and 0.007%, respectively).

The pre-transplant PB sample of UPN2 (VEXAS and Intermediate IPSS-R MDS) showed a 95% $UBA1^{mut}$ VAF, which dramatically decreased at + 30 days after allo-HCT (0,59%), at + 100 days (0,17%), until complete disappearance at +180 and +365 days (VAF 0% in both determinations).

UPN3 had VEXAS and associated MDS and evolved to AML, as previously described.(15) Briefly, the patient was treated with azacitidine-venetoclax but due to infectious complications temporarily stopped treatment and

eventually relapsed. We here describe his follow-up: upon improvement of the performance status, chemotherapy with 3+7 was started as a bridge to allo-HCT with rapid decrease of *UBA1* VAF from 23,9% before 3+7 chemotherapy, to 0,01% at +5 months from the transplant procedure.

UPN4 was a patient with VEXAS and associated low IPSS-R MDS for whom treatment with erythropoietin did not modify *UBA1* VAF (78,1% at +4 years of therapy, VAF 66,5% at + 8 years).

Discussion

In this study, we leveraged the Italian network of rheumatologists and hematologists to explore the diagnostic capabilities and clinical-genomic characteristics of VEXAS after 2.5 years since its first description.(1) Prompted by the novelty of the disorder and the lack of official guidelines, this nationwide survey has allowed us to gather information on techniques currently used to identify *UBA1* mutations, the clinical and genomic characteristics of patients with VEXAS diagnosed so far in Italy, and to investigate the different therapeutic approaches used. We also tracked longitudinal *UBA1* clonal dynamics upon a variety of treatments, confirming their disease-modifying capabilities, and potentially demonstrating its utility in the future for guiding therapeutic decisions and disease monitoring.

At least half of the responding centers had available *UBA1* diagnostics, with Sanger sequencing of exon 3 (location of the hotspot Met41(1, 22)) as the most common used method. As *UBA1* mutations are usually present at high VAFs[21], Sanger sequencing may be a rapid and quite inexpensive first screening in highly suspicious patients.(5) However, new mutations have been defined in patients negative for canonical exon 3 variants and screened with other techniques(23) such as whole genome sequencing or WES(17), as also exemplified in our cohort. Therefore, while Sanger can be used in the first place, negative cases highly suspicious for VEXAS are the ideal candidates for *UBA1* whole gene sequencing methods.

In line with previous literature(24), our patients displayed a variety of clinical manifestations, with some being more constant and recurrent than others, namely BM vacuoles, male gender, and systemic inflammation. However, the heterogeneity of possible clinical scenarios poses challenges in disease recognition and clinical management, which is also hindered by the multidisciplinary nature of the condition.(8) In light of this, we showed

that a combination of competences across different specialties may guide clinical identification of suspected cases, and their management in an individualized fashion, while waiting for official disease guidelines. It is then unsurprising that in our cohort patients were treated with a variety of rheumatologic and hematologic therapies with a median of 3 lines of treatments. Specific strategies have also been used in case of particular disease manifestations. For instance, patients with thrombosis required anti-coagulation, while 3 cases with concomitant MDS received allo-HCT. This is the only curative approach able to substitute the *UBA1*-mutant clonal hematopoiesis and, as a result, abate the clinical manifestations of the disease, and anecdotal reports described its utility in this setting.(13, 25, 26) Nonetheless, allo-HCT is burdened by a non-negligible mortality risk, and should be judiciously pondered in patients such as those with VEXAS, typically old (median age in our cohort was 67 years) and with a multitude of clinical conditions posing specific transplant challenges. Remarkably, we showed that in all 3 cases allografted in our cohort, allo-HCT reverted the VEXAS clinical phenotypes and parallelly cleared *UBA1* mutations. MDS/AML-directed approaches also decreased *UBA1* VAF, paralleling clinical disease remissions as exemplified by UPN3. Conversely, we showed that ruxoltinib had only a suspensive effect on VEXAS clinical manifestations while causing an increase of *UBA1* clonal burden in UPN1. This is in line with Heiblig et al(27) who showed that, after treatment with ruxolitinib, the *UBA1* clone completely overwhelmed the myeloid neoplastic clone in 2 VEXAS patients with respectively chromosome 7 deletion and *JAK2* mutation.(27) Finally, supportive therapy with growth factors as erythropoietin did not alter *UBA1* kinetics, as exemplified by UPN4.

Over the last decade, the link between BM aging and somatic mutations has been typified by the discovery of clonal hematopoiesis of indeterminate potential (CHIP) in healthy, elderly individuals.(28-31) The aging component is so relevant to this process that another acronym, ARCH (age-related clonal hematopoiesis) has been coined to refer to the specific acquisition of mutations in myeloid genes, typically of the DAT (*DNMT3A, ASXL1, TET2*) triad, in elderly individuals.(32) A growing line of research has then demonstrated the link between occurrence of CHIP/ARCH and inflammation, with a known risk for carriers not only of myeloid neoplasia, but also of cardiovascular events and increased all-cause mortality.(33) Obvious are the mechanistic analogies of the above-mentioned conditions to VEXAS syndrome. Indeed, this is the first condition encompassing

CHIP/ARCH, "inflamm-aging" and MDS all together under the same nosological entity.(3) In our cohort, more than two third of patients had concomitant MDS, and typically presented with lower IPSS-R/M risk profiles, as substantiated by the low mutational burden with a landscape characteristics of ARCH. This finding is in line with a recent report exploring the spectrum of clonal hematopoiesis associated with VEXAS by means of prospective single-cell proteogenomic sequencing.(34) This study showed that *DNMT3A* mutations ontogenetically preceded *UBA1* alterations, whereas *TET2* and other gene mutations occurred subclonally to *UBA1* or as independent clones.(34, 35) It is noteworthy to mention that we previously reported the first case of VEXAS/MDS evolving to AML, and here we describe a second case.(15) Both patients did not carry *DNMT3A* mutations, perhaps arguing for clonal trajectories of these progressive cases more similar to that of general MDS than of *UBA1/DNMT3A*-mutant VEXAS.(35) The latter may be instead the culmination of "inflamm-aging" forces driving both ARCH and then *UBA1* mutational acquisition. (3)

In conclusions, we here present the Italian VEXAS experience taking advantage of a multidisciplinary network of specialists, detailing the heterogeneous clinical and genomic characteristics of cases. Patients required a variety of rheumatologic treatments and exhibited concomitant MDS in roughly 70% of cases. By tracking longitudinal clonal dynamics, we were able to show *UBA1* clonal burden variations under different therapeutic exposures including allo-HCT, the only treatment able to eradicate *UBA1* mutations, cure the disease and currently explored in a phase II trial (NCT05027945). Follow-up to this study would be useful to identify the effects of the various treatments on the clonal dynamics of the *UBA1* gene to spot potential prognostic factors and accurately investigate minimal residual disease (MRD)-directed therapeutic strategies.(13)

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Tables

Table 1. Clinical characteristics of patients with VEXAS syndrome (n=41).

Characteristics	
Male gender, n (%)	41 (100)
Age in years at diagnosis, median (IQR)	67 (62-72)
Chondritis, n (%)	20 (49)
Skin lesions, n (%)	14 (34)
BM vacuoles*, n (%)	32 (89)
Laboratory data	
Hemoglobin (g/dl), median (IQR)	9.1 (8-11)
Platelets (x 10^9/L), median (IQR)	127,000 (87,000-172,000)
Leucocytes (x 10^9/L), median (IQR)	3,800 (2,540-5,900)
Neutrophils (x 10^9/L), median (IQR)	2,020 (1,200-3,700)
Unprovoked thrombosis*, n (%)	11 (28)
VEXAS therapy*	1
No treatment, n (%)	3
Steroids**, n (%)	33 (83)
DMARDs**, n (%)	20 (50)
Azacitidine**, n (%)	3 (8)
Concomitant hematological disorders*	
MM, n (%)	0
MGUS, n (%)	15 (37)
MDS, n (%)	28 (71)
Progression MDS to AML, n (%)	2 (7)
Deaths, n (%)	5 (12)

*Missing data, percentage calculated on cases with available information; **ever received.

Figure legends

Figure 1. Italian cartography of VEXAS and clinical manifestation. A) Map of Italy highlighting the centers that had available *UBA1* genomic testing. B) A bar chart showcases the diagnostic techniques used in the Italian centers. C) May Grünwald-Giemsa staining of bone marrow aspirate from a representative patient with VEXAS depicting the phenomenon of vacuolization of the hematopoietic precursors. D): Clinical manifestations of VEXAS in our cohort.

Figure 2. VEXAS associations and outcomes. Panel A) shows a pie chart with patients with VEXAS syndrome and presence of MDS classified in accordance with guidelines from the World Health Organization (WHO) 2022. Panel B) shows IPSS-R e IPSS-M distribution of cases with MDS-associated VEXAS syndrome (percentages are calculated on cases with available information). Panel C) Kaplan Meier curve and 95% confidence interval for the entire cohort. Numbers at risk are indicated below the curve.

Figure 3. *UBA1* clonal dynamics upon different therapeutics. Graphs showing the clonal dynamics of the *UBA1* gene, studied by means of ddPCR, in 4 patients who underwent different treatment approaches.