Received: 17 February 2021 | Revised: 7 May 2021 | Accepted: 10 May 2021

DOI: 10.1002/ajh.26234

RESEARCH ARTICLE

AJH WILEY

Natural gene therapy by reverse mosaicism leads to improved hematology in Fanconi anemia patients

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Funding information
Agencia Estatal de Investigación, Grant/Award Numbers: CB06/07/0023, RTI2018-098419-B-I00, Red2018-102372;

Abstract

Fanconi anemia (FA) is characterized by chromosome fragility, bone marrow failure (BMF) and predisposition to cancer. As reverse genetic mosaicism has been described as "natural gene therapy" in patients with FA, we sought to evaluate the clinical course of a cohort of FA mosaic patients followed at referral centers in Spain over a 30-year period. This cohort includes patients with a majority of T cells without

Abbreviations: AML, Acute myelogenous leukemia; BFU-E, Burst forming unit-erythroid; BM, Bone marrow; BMF, Bone marrow failure; CFCs, Colony forming cells; CFU-GEMM, Colonyforming Unit-Granulocyte, Erythrocyte, Monocyte/macrophage, Megakaryocyte; CFU-GM, Colony forming unit-granulocyte and monocyte; DEB, Diepoxybutane; FA, Fanconi anemia; HSCT, Hematopoietic stem cell transplant; HSPCs, Hematopoietic stem and progenitor cells; ICL, Interstrand cross-link; MDS, Myelodysplastic syndrome; MMC, Mitomycin-C; WES, Whole exome sequencing.

María José Ramírez and Roser Pujol contributed equally to this work.

European Commission, Grant/Award Numbers: EUROFANCOLEN project HEALTH-F5-2012-305421, P-SPHERE COFUND project; Fanconi Anemia Research Fund; Fundación Ramón Areces; Generalitat de Catalunya, Grant/Award Number: CERCA Programme; ICREA-Academia program; Instituto de Salud Carlos III, Grant/Award Number: CIBERER intramural projects; Ministerio de Sanidad, Servicios Sociales e Igualdad, Grant/Award Numbers: FANCOLEN, FANCOSTEM

chromosomal aberrations in the DEB-chromosomal breakage test. Relative to non-mosaic FA patients, we observed a higher proportion of adult patients in the cohort of mosaics, with a later age of hematologic onset and a milder evolution of (BMF). Consequently, the requirement for hematopoietic stem cell transplant (HSCT) was also lower. Additional studies allowed us to identify a sub-cohort of mosaic FA patients in whom the reversion was present in bone marrow (BM) progenitor cells leading to multilineage mosaicism. These multilineage mosaic patients are older, have a lower percentage of aberrant cells, have more stable hematology and none of them developed leukemia or myelodysplastic syndrome when compared to non-mosaics. In conclusion, our data indicate that reverse mosaicism is a good prognostic factor in FA and is associated with more favorable long-term clinical outcomes.

1 | INTRODUCTION

Fanconi anemia (FA) is a rare inherited bone marrow failure (BMF) syndrome that results from single or compound mutations in any one of the 22 known genes encoding for proteins involved in the FA/BRCA DNA repair pathway. This pathway is comprised of core, ID, and effector complexes responsible for repair of DNA damage resulting from interstrand cross-link (ICL) lesions, leading to stalled DNA replication forks.[1–6] Defective DNA repair leads to impaired bone marrow (BM) function, cytopenia and, ultimately, BMF, which takes place in 80% of FA patients during the first decade of life.[7] In addition to progressive BMF and diverse congenital abnormalities, FA is also characterized by cancer predisposition. The FA patients commonly develop hematologic malignancies, particularly myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) with a cumulative incidence of MDS/AML of approximately 20% by age 20 and 30% by age 30.[7] The incidence of solid tumors similarly increases with age and in recent series approached 40% by age 40.[8]

Somatic mosaicism in hematopoietic cells, often described as "natural gene therapy," is a phenomenon that occurs in a subset of FA patients and derives from a reversion or other compensatory mutation in hematopoietic cells restoring the FA/BRCA pathway. In mosaic patients, there is frequently a population of hematopoietic cells that is sensitive to ICL DNA-damaging agents and a distinct population resistant to these drugs.[9–11] Cases have been reported in which mosaicism has led to spontaneous correction of BMF, at times resulting from an apparent reversion or compensatory mutation in hematopoietic stem and progenitor cells (HSPCs).[11–14] A recently published retrospective review of literature-reported FA mosaicism cases has indicated that mosaicism may be associated with lower incidence of BMF or hematologic malignancy, lower requirement for allogeneic hematopoietic stem cell transplant (HSCT), and relatively lower mortality during the initial 2–4 decades of life[15] compared to non-mosaic FA patients. In the current retrospective study and in order to further understand the prognostic implications of mosaicism in FA, we sought to evaluate the clinical course of a cohort of FA mosaic patients followed at referral centers in Spain over a 30-year period. Clinical phenomena of interest included the development of BMF, malignancy, requirement for allogeneic HSCT, survival and cause of death in order to further elucidate the prognostic implications of mosaicism in FA.

We further sought to understand the clinical course of a smaller subset of patients with documented ICL-resistance in BM progenitor cells, by means of mitomycin-C (MMC)-resistance of BM colony forming cells (CFCs). Because compensatory mutations may develop in both pluripotent and more committed progenitor lineages, the presence of diepoxybutane (DEB)-resistant T-lymphocytes (measured as part of a standard FA-diagnostic evaluation) may not be indicative of multilineage hematopoietic resistance. The presence of MMC-resistance within BM CFCs has been hypothesized as potentially more indicative of comprehensive genetic reversion and concomitant protection of the hematologic consequences of FA. However, this test is not routinely performed in the clinic and it has not been consistently used in the classification of patients with FA. In contrast, information regarding DEB-resistance in T-lymphocytes was available for all Spanish patients with FA.

2 | METHODS

2.1 | Patients

The national registry of patients with FA from Spain was created in 1998. Patients with FA were diagnosed based on clinical findings and positive results from DEB T-lymphocyte chromosomal fragility test. The diagnosis of FA in patients with a high degree of mosaicism at presentation was confirmed by a chromosome breakage test in skin fibroblasts or by mutational analysis (data not shown). Clinical data from patients with FA were obtained from their clinicians. All relevant ethical regulations were applied and informed consent was obtained from all participants.

Mosaic FA patients were identified from a cohort of 223 Spanish FA patients (data collected up to September 2020) from the national registry based on peripheral blood DEB T-lymphocyte chromosomal fragility test. Historically there has been no evident threshold level for

the percentage of aberrant cells that defines mosaicism. Potentially significant threshold ranges have been described in previous papers[9,16] and based on these and on the distribution of aberrant cell percentages in Spanish FA patients, a threshold was defined. This distribution does not fit within a Gaussian distribution (Kolmogorov–Smirnov test $p = < .001$) and has a Q1 = 52%, a Q3 = 84% and a median percentage of aberrant cells of 72% (Figure S1). In accordance, we have categorized patients with a majority of T cells without DEB-induced chromosome breaks in peripheral blood as T-cell mosaics. These patients have less than 50% of T-lymphocytes with chromosomal aberrations, noting that this value is below the Q1 of the distribution of aberrant cells in patients with FA. When the cut-off of 50% of aberrant cells is used to differentiate mosaic vs non-mosaic patients, the distribution of both populations displays a Gaussian distribution (Figure 1(A)). Patients with more than 50% of T-lymphocytes with DEB-induced chromosomal aberrations were considered as non-mosaic FA patients. For the smaller sub-population of mosaic patients whose BM had been evaluated for MMC-resistance, a classification of myeloid mosaicism was attributed if resistant colonies were observed after culture in the presence 10 nM of MMC. Patients with mosaicism in both T cell and myeloid lineages were considered as multilineage mosaic patients.

A database was constructed to evaluate clinical features including age at FA and mosaic diagnoses, duration of follow-up, development of BMF, malignancy, allogeneic HSCT, survival and cause of death. Additionally, laboratory assessments such as blood cell counts and T-lymphocyte and BM CFC resistance testing were also assessed.

2.2 | DEB T-lymphocyte chromosomal fragility test

Chromosome fragility tests on peripheral blood lymphocytes were performed as described previously.[9,17] Blood cultures were prepared for each patient and were stimulated with phytohemagglutinin. Twenty-four hours later, samples were treated with or without DEB for 46 h. Metaphase spreads were obtained according to standard cytogenetic methods and finally, stained with Giemsa. For chromosome fragility evaluation, 25–50 metaphases with 46 chromosomes were analyzed for each culture. The microscopic analysis was performed with a Leitz Aristoplan microscope or with a Zeiss Imager M1 microscope coupled to a computer assisted metaphase finder (Metasystems, Werfen S.A.U., Barcelona, Spain).

2.3 | CFC assays

The CFCs were analyzed considering total CFCs including colony forming unit-granulocyte and monocyte (CFU-GM) and burst forming unit-erythroid (BFU-E). Colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte (CFU-GEMM) were not identified separately. Erythroid cells were depleted from total BM with HES (Grifols Laboratories, Barcelona, Spain) as previously described.[18] So, 2.5×10^5 erythroid depleted BM cells were plated in triplicate in methylcellulose medium (MethoCult H4434; Stem Cell Technologies, Grenoble, France) supplemented with 10 μg/ml anti-TNFα and 1 mM N-acetylcysteine. Cells were incubated for 14 days at 37°C in 5% O_2, 5% CO_2 and 95% humidified air. The proportion of MMC-resistant CFCs was calculated based on colony numbers scored in the absence and presence of 3 and 10 nM MMC (Sigma–Aldrich, Madrid, Spain).

2.4 | Subtyping by complementation studies with retroviral vectors

Retroviral subtyping on blood T cells was based on the reversion of MMC hypersensitivity of FA cells mediated by the transfer of complementary FA genes, using retroviral vectors as described previously.[19] Briefly, mononuclear cells from heparinized peripheral blood were stimulated and infected with retroviral supernatants. After that, cells were exposed to increasing concentrations of MMC (0–1000 nmol/L). Five days later, cell viability was determined by flow cytometry (LSRFortessa cell analyzer, BD/Becton, Dickinson and Company, New Jersey, USA) based on the DAPI (4′,6-diamino-2′-phenylindole, dihidrocholride) exclusion fluorescence.

2.5 | Whole exome sequencing (WES)

Whole exome sequencing was performed as described previously.[20] Briefly, DNA samples from FA patients were from peripheral blood, primary fibroblast and Epstein–Barr virus immortalized lymphoblastoid cell lines. Sanger sequencing following target PCR amplification was used for validation of each variant. Whole exome sequencing analysis was performed by Eurofins Genomics, Ebersberg Germany (InView Human Exome).

2.6 | Statistics

Means were statistically compared using a Mann–Whitney non-parametric test for independent samples. Percentage distributions were statistically compared using a chi-square test. Survival curves were compared using a Log-rank (Mantel-Cox) test. The fit with a Gaussian distribution was evaluated with a Kolmogorov–Smirnov test. Statistical analyses were performed using GraphPad Prism software.

3 | RESULTS

3.1 | Mosaic patient characteristics

Based on the aforementioned criteria, 41 patients (18%) from the total cohort of 223 FA patients were identified as mosaics. Of these 41 patients, 34 were initially diagnosed as T-cell mosaics (less than 50% of aberrant T cells following DEB treatment), and the remaining

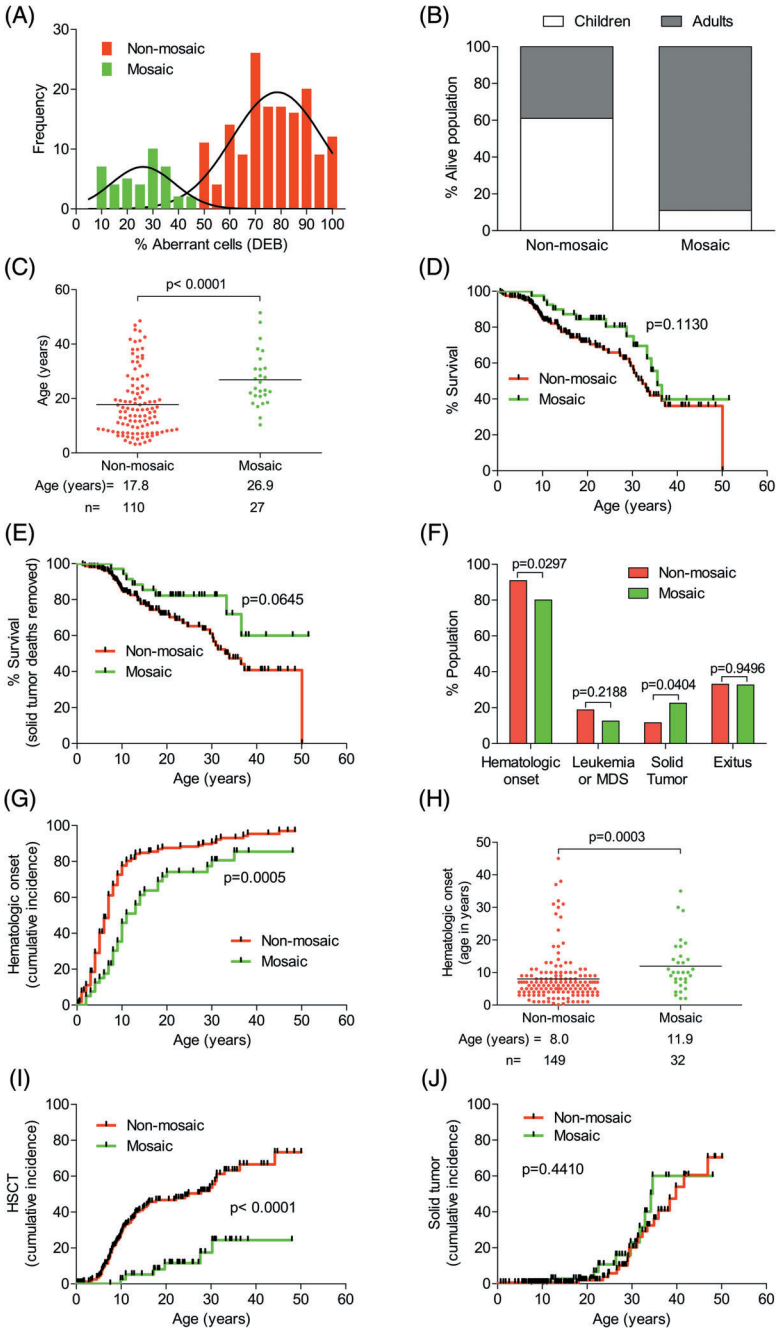

FIGURE 1 Different distribution between non-mosaic and mosaic patients in percentage of aberrant cells, hematologic onset, stem cell transplantation and solid tumors but similar long-term survival. (A) Distribution of the percentage of aberrant cells in mosaics and non-mosaics FA patients. The cut off of 50% of aberrant cells is used to differentiate mosaic vs non-mosaic patients, when this cut off is used the distribution of both populations fits with the Gaussian distribution (Kolmogorv-Smirnov test, $p = .137$ and $p = .050$ in mosaic and non-mosaic population respectively). Mosaic population has a Q1 of 15.5% and a Q3 of 34% of aberrant cells with a median of 28% and the non-mosaic population has an Q1 of 68% and a Q3 of 88% of aberrant cells with a median of 76%. (B) Percentage distribution of children and adults in non-mosaic and mosaic patients who were alive at time of analysis. Eighty-nine percent of mosaic patients reached adulthood, compared to only 39% of non-mosaic patients (chi-square, $p < .001$), indicating that the probability of mosaicism is substantially higher in the subgroup of adult FA patients. (C) Mosaic patients have a mean age higher than non-mosaic patients (26.9 vs 17.8 years, $p < .001$; means were statistically compared using a non-parametric Mann–Whitney test). (D) Similar overall survival of mosaic vs non-mosaic patients ($p =$ h .113; curves were compared using a Log-rank (Mantel-Cox) test). (E) Overall survival when solid tumors are removed as a cause of death is better in mosaic patients ($p = .065$; curves were compared using a Log-rank (Mantel-Cox) test). (F) Disease manifestations in non-mosaic vs mosaic patients. For hematologic onset, 91% of non-mosaic and 80% of mosaics have abnormal blood counts ($p = .030$); 12% of non-mosaic and 23% of mosaics were affected with solid tumors ($p = .040$). Comparisons between non-mosaic and mosaic patients were done using a chi-square test. (G,H) Later onset of blood disease in mosaic FA patients, as seen in cumulative incidence (G), $p < .001$ curves were compared using a Log-rank (Mantel-Cox) test) and age distribution (H), $p < .001$; means were statistically compared using a non-parametric Mann–Whitney test). (I) Higher cumulative incidence of HSCT in non-mosaic vs mosaic FA patients ($p < .001$; curves were compared using a Log-rank (Mantel-Cox) test). (J) Equal incidence of solid tumor between mosaic and non-mosaic patients. Curves were compared using a Log-rank (Mantel-Cox) test ($p = .441$) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Main clinical data of mosaic FA patients

Code FA	Sex	Gene	Age patient (years)	Years follow-up	% Aberrant cells (at diagnosis)	% Aberrant cells (became mosaic)	Age diagnosis (years)	Age onset hematological failure (years)	Alive/ Exitus	HSCT	Leukemia/ MDS	Solid tumor
FA0005	F	*FANCA*	35	30	20	At diagnosis	11	8	Alive	No	No	Yes
FA0029	F	*FANCT*	31	11	15	At diagnosis	8	No onset	Alive	No	No	No
FA0049	M	*FANCA*	28	28	34	At diagnosis	2	2	Alive	Yes	No	No
FA0052	M	*FANCA*	34	27	26	At diagnosis	7	6	Exitus	No	No	Yes
FA0055	M	*FANCA*	21	18	78	28	0	8	Alive	No	No	No
FA0078	F	*FANCD2*	11	1	32	At diagnosis	10	10	Exitus	Yes	No	No
FA0090	F	*FANCA*	31	17	15	At diagnosis	14	14	Alive	No	No	No
FA0094	F	*FANCA*	10	6	36	At diagnosis	9	5	Exitus	No	No	No
FA0113	F	*FANCA*	12	4	46	At diagnosis	9	9	Exitus	No	Yes	No
FA0121	M	*FANCE*	26	20	18	At diagnosis	0	No onset	Unknown	No	No	No
FA0124	F	*FANCD2*	18	8	26	At diagnosis	13	10	Alive	No	No	No
FA0125	F	*FANCA*	23	18	10	At diagnosis	4	7	Alive	No	No	No
FA0126	M	*FANCA*	19	18	69	10	2	4	Alive	No	No	No
FA0153	M	*FANCA*	17	7	36	At diagnosis	13	4	Exitus	No	Yes	No
FA0157	F	*FANCA*	15	4	36	At diagnosis	9	Unknown	Exitus	No	No	Yes
FA0158	M	*FANCD1*	8	6	38	At diagnosis	2	No onset	Exitus	No	No	No
FA0187	M	*FANCA*	42	24	10	At diagnosis	1	29	Alive	Yes	Yes	No
FA0235	M	*FANCA*	31	10	29	At diagnosis	3	No onset	Alive	No	No	No
FA0294	F	*FANCA*	38	16	22	At diagnosis	26	20	Alive	Yes	No	No
FA0331	M	Unknown	23	12	15	At diagnosis	12	3	Alive	No	No	No
FA0342	M	*FANCD2*	24	14	34	At diagnosis	10	10	Alive	No	No	No
FA0344	F	*FANCD2*	21	14	68	32	8	9	Alive	No	No	No
FA0383	F	*FANCD2*	27	13	78	44	14	14	Alive	No	No	No
FA0409	M	*FANCA*	33	2	28	At diagnosis	10	11	Exitus	No	No	Yes
FA0434	M	*FANCA*	10	9	74	9	1	10	Alive	Yes	No	No
FA0438	M	*FANCA*	21	12	60	38	9	11	Alive	No	No	No
FA0550	F	*FANCA*	29	4	11	At diagnosis	8	8	Exitus	No	Yes	Yes
FA0553	M	*FANCA*	30	2	10	At diagnosis	28	13	Exitus	No	No	Yes
FA0554	M	*FANCA*	24	6	32	At diagnosis	12	18	Exitus	No	No	Yes
FA0558	M	*FANCA*	13	10	70	18	3	2	Alive	No	No	No
FA0568	F	*FANCA*	18	17	27	At diagnosis	10	7	Alive	No	No	No
FA0574	M	*FANCB*	28	13	21	At diagnosis	18	18	Alive	No	No	No

(Continues)

TABLE 1 (Continued)

Code FA	Sex	Gene	Age patient (years)	Years follow-up	% Aberrant cells (at diagnosis)	% Aberrant cells (became mosaic)	Age diagnosis (years)	Age onset hematological failure (years)	Alive/ Exitus	HSCT	Leukemia/ MDS	Solid tumor
FA0663	F	*FANCD1*	36	6	32	At diagnosis	33	No onset	Exitus	No	No	Yes
FA0664	M	*FANCD2*	23	7	19	At diagnosis	15	No onset	Alive	No	No	No
FA0681	M	*FANCD2*	26	7	26	At diagnosis	19	19	Alive	No	No	No
FA0707	F	*FANCA*	22	6	28	At diagnosis	16	15	Alive	Yes	Yes	No
FA0775	M	*FANCA*	48	7	40	At diagnosis	43	No onset	Alive	No	No	No
FA0829	M	*FANCA*	38	2	12	At diagnosis	36	No onset	Alive	No	No	No
FA0850	F	*FANCA*	37	4	36	At diagnosis	36	35	Exitus	No	No	Yes
FA0875	F	*FANCA*	17	3	34	At diagnosis	13	13	Alive	No	No	No
FA0993	F	Unknown	52	0	32	At diagnosis	51	30	Alive	Unknown	Unknown	Unknown
Mean			26	11	34	26	13	12				

Abbreviations: F, female; HSCT, hematopoietic stem cell transplant; M, male; MDS, myelodysplastic syndrome.

seven were initially diagnosed as non-mosaic (more than 50% of aberrant cells) and evolved to mosaic during the follow-up (Table 1) indicating the dynamic process of mosaicism. In our population we did not observe any patient who was mosaic at the time of diagnosis and who subsequently evolved to a non-mosaic state. The average follow-up period of these 41 patients was 11 years, ranging from 0–30 years. Of these 41 mosaic patients, 19 (46%) were female and 22 (54%) were male (Table 1).

3.2 | Complementation groups

Complementation groups in the mosaic cohort were *FANCA* (n = 27; 66%), *FANCD2* (n = 7; 17%), *FANCD1* (n = 2; 5%), *FANCB* (n = 1; 2%), *FANCT* (n = 1; 2%), *FANCE* (n = 1; 2%), and two with unknown complementation group (Table 1). In addition to identify the affected FA gene, we categorized the specific mutations involved (Table S1) in order to understand if some mutations are more likely to revert and consequently are more frequently represented in the mosaic population.

3.3 | Mosaic patient outcomes

3.3.1 | Overall survival

Eighty-nine percent of the mosaic patients reached adulthood (18 years or older) whereas only 39% of non-mosaic patients did so (chi-square, $p < .001$) (Figure 1(B); Table S2). Additionally, mosaic patients had a mean age of 26.9 years (n = 27 alive patients) vs 17.8 years of the non-mosaic patients (n = 110 alive patients) (Figure 1(C)). At the time of last follow-up, a similar proportion of living patients was observed in the mosaic (68%) and non-mosaic (67%) cohort (Figure 1(D) and Table S2). However, the cumulative incidence of alive patients in the mosaic cohort improved compared to that of the non-mosaic cohort when solid organ malignancies were removed as a cause of death (Figure 1(E)).

3.3.2 | Hematologic manifestations

Hematologic follow-up of the mosaic patients indicates that 72% had normal (platelets>147×10^9 cells/L) or stable platelet levels (platelets<147×10^9 cells/L but without requirement for transfusions or HSCT) at the time of this study (Table S3). Although most of non-mosaic (91%) and mosaic patients (80%) had hematologic disease onset (IFAR, International Fanconi Anemia Registry, defines onset of hematological manifestations as at least one of the following blood counts: ANC < 1000/μl, Hb < 100 g/L, platelets < 100,000/μl, or development of MDS or AML), these percentages were statistically different (Figure 1(F), $p = .030$), and mosaic patients had a later hematologic disease onset as demonstrated by cumulative incidence (Figure 1(G), $p < .001$) and age distribution (Figure 1(H), $p < .001$). At

7 years of age, 61% of non-mosaic patients had developed signs of hematologic abnormalities and only 23% of mosaic patients had signs of hematologic abnormalities. More specifically, the mean age of hematologic disease onset in mosaic patients was 12 years compared to 8 years in non-mosaic patients. Interestingly, a lower requirement for HSCT in mosaic vs non-mosaic patients was observed ($p < .001$; Figure 1(I)); at 18 years of age, 47% of non-mosaic patients required HSCT vs only 8% of mosaic patients.

Prolonged hematologic stability over several decades has been seen in mosaic patients. Patient FA0005 has had stable peripheral blood counts over 30 years with hemoglobin and platelet counts consistently above standard transfusion thresholds (Figure 2).

3.3.3 | Solid tumor prevalence

The prevalence of solid tumors was higher in mosaic patients at 23% (vs. 12% in non-mosaic patients) likely secondary to the older age of mosaic patients (Figure 1(F)). The effect of age on the incidence of solid tumors is statistically confirmed using a univariate General Lineal Model with a clear effect of older age on the incidence of solid tumors ($p < .001$) independent of mosaicism status ($p = .614$). Additionally, an equal incidence of solid tumors between mosaic and non-mosaic patients was found when the cumulative incidence of solid tumors was compared in both populations using a Log-rank (Mantel-Cox) test ($p = .441$, Figure 1(J)). The higher incidence of solid tumors in mosaic patients is observed mainly in non-transplanted patients, probably due to the fact that the number of transplanted patients in this cohort is low, only six out of a total of 40 patients for whom we have transplant data (Table S4).

3.3.4 | Multilineage mosaics

Data on BM CFC MMC survival was available from 11 out of 41 mosaic patients (Tables S5 and S6; Figure 3(A)). Survival data were obtained considering total CFCs including CFU-GM and BFU-E (CFU-GEMM were not identified separately). A threshold to define a myeloid mosaic was established taking into account that mosaicism is a dynamic process and that the chronologic interval between hematopoietic stem cell reversion and an observable subsequent hematologic effect is unknown. It is important to note that FA patients without mosaicism usually do not show BM MMC resistant colonies; consequently the finding of some resistant colonies is an indication that some HSPCs have reverted and that therefore, in a strict sense, this individual is mosaic in BM. However, the type and number of reverted cells and the clonal expansion of these cells will determine the improvement in the patient's hematology. Thus, it is highly important to establish a threshold for percentage of resistance to define myeloid mosaicism. Given the lack of previous data in this regard, we established the threshold of resistance according to our data. In our experience, with 11 patients studied for CFC resistance to MMC and nine of these patients with some resistant colonies, we observed a median percentage of MMC resistant colonies of 31% when the 11 patients are included in the distribution; and a median of 46% when only patients with resistant colonies are included. According to this distribution, we would suggest a threshold to consider FA patients as myeloid mosaics when the percentage of MMC resistant colonies was above 14% (above Q1 when the 11 patients are studied). Additionally, we observed some hematologic improvement when this percentage was above 21% (above Q1 when only patients with resistant colonies are studied), suggesting that a minimum number of reverted BM cells are needed to revert the hematologic failure in FA patients. Taking into account the threshold of 14% of MMC resistant colonies nine out 11 patients (82%) were classified as multilineage mosaics because they were mosaics in the T-cell lineage (less than 50% of aberrant cells in peripheral blood after DEB treatment) as well as in the myeloid lineage (resistant colonies in BM after MMC treatment). Two patients did not show BM CFC MMC resistance and were classified as specific T-cell mosaics. The mean age of the multilineage mosaics was 24 years old, while the mean age of those with specific mosaicism in T-cell lineage was 21 years old. Seven out of nine (78%) patients with myeloid mosaicism had stable hematology (within normal limits or if below not requiring transfusions or HSCT) and none presented with leukemia or MDS. An example of multilineage mosaic is patient FA0005 who has had over 30 years of hematologic stabilization (Figure 2). As indicated in Figure 3(B), all patients with unstable hematology had less than 20% of BM CFCs resistant to MMC (10 nM). Importantly, when solid organ cancer was removed as a cause of death 83% of the subgroup of multilineage mosaics were alive at the age of 34.6 (Figure 3(C)) and at the same age, these patients had only an 11% of cumulative incidence of HSCT (Figure 3(D)). No correlation was observed between the percentage of MMC survival of CFCs and the percentage of aberrant cells in peripheral blood after DEB treatment ($p = .891$). This comparison was done only in six patients whose tests were performed on the same date or with a difference of less than 5 months due to the dynamism in the mosaicism process.

According to our data, nine out of 11 patients with mosaicism in T cells also showed myeloid mosaicism, suggesting that approximately 82% of T-cell mosaics according to the defined in this study are multilineage mosaic patients.

4 | DISCUSSION

Using the diagnostic criterion for mosaic patients developed in this paper, we observed that 18% of patients with FA are mosaic, in the range of the published incidence of mosaicism in FA of 15%–25%.[11,14] This diagnostic criterion allows for detection of multilineage mosaics (involving myeloid and lymphoid lineage), mosaics in only lymphoid lineage (B and T mosaics) and mosaics exclusively in the T-

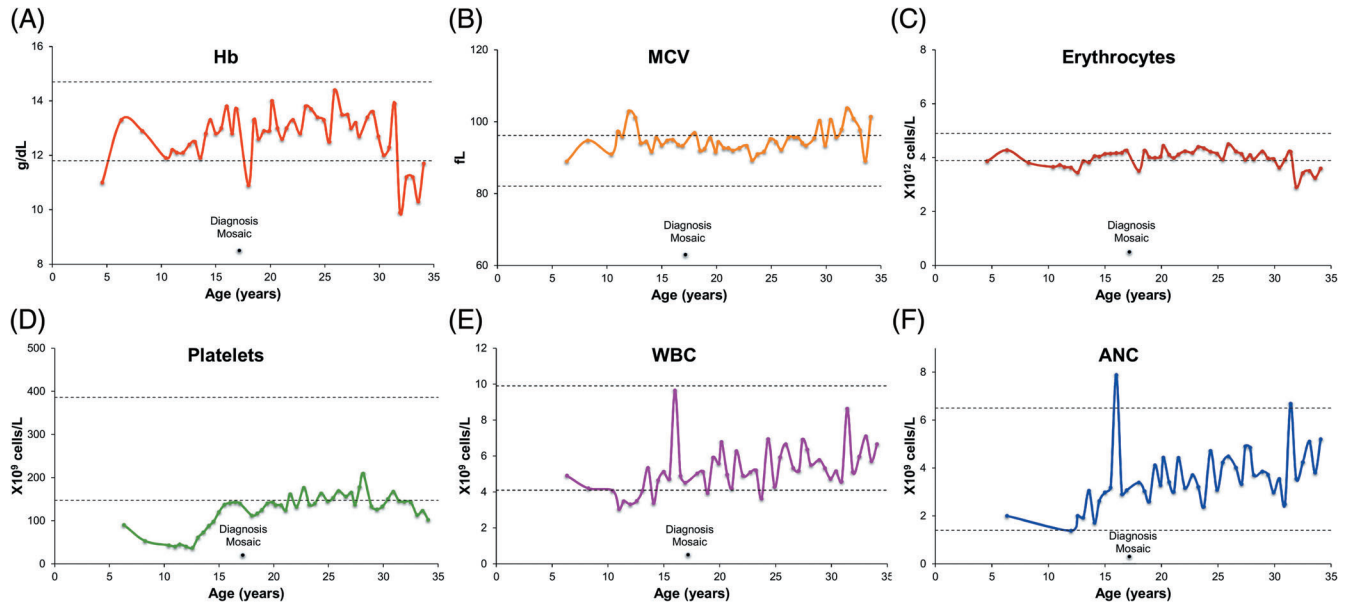

FIGURE 2 Long term follow-up of hematologic parameters in a multilineage mosaic (myeloid and lymphoid lineage) patient FA0005. Hematologic data are represented according to the patient's age expressed in years. FA0005 patient has a genetic reversion in BM and peripheral blood, with stable hematology (still alive at age 34 years). (A) Hemoglobin (Hb) in g/dl. (B) Mean corpuscular volume of erythrocytes (MCV) in fl. (C) Erythrocytes in $\times 10^{12}$ cells/L. (D) Platelets in $\times 10^{9}$ cells/L. (E) Leukocytes (WBC) in $\times 10^{9}$ cells/L. (F) Neutrophils (ANC) in $\times 10^{9}$ cells/L [Color figure can be viewed at wileyonlinelibrary.com]

lymphoid lineage (T-cell mosaics). However, mosaics exclusively in the myeloid lineage or only B-lymphoid lineage are not detectable with the DEB test in peripheral blood, because only Tcells are studied. Consequently, the total number of mosaics of all types is underestimated with this method.

Among the 41 mosaic patients identified in this analysis, only 11 were evaluated for MMC resistance in BM CFCs. Among these, nine showed MMC-resistant colonies, indicating that the reversion appeared in a HSPC capable of giving rise to both T-cell and myeloid lineages (multilineage mosaic). The remaining two did not show MMC-resistant colonies, indicating that stem or progenitor cells had not reverted, and consequently, the reversion appeared in a more committed lineage, producing T cells but not cells of the myeloid lineage. Therefore, among 41 mosaic patients, we determined that two are mosaics only in T-cell lineage, nine are multilineage mosaics (lymphoid and myeloid lineage) and the remaining 30 are at least T-cell mosaic patients. In the absence of BM clastogen-resistance data, it is not possible to know if the reversion affected the other lineages. It is important to recognize that some patients who were classified as non-mosaic by the DEB test in peripheral blood could be mosaics only in the myeloid lineage with MMC resistant CFCs in BM and with a stable hematology. Although the BM CFC MMC resistance testing is not a universally performed clinical test, the data suggest that marrow CFC resistance may have important prognostic implications, especially, when the percentage of resistant CFCs in the presence of MMC (10 nM) is approaching or above 20%, based on our data in nine patients with resistant colonies. This threshold, approximately equivalent to the first quartile (21%) when considering the nine multilineage mosaic patients could indicate that a percentage of reverted HSCs in this range is necessary to enable a sustained hematologic improvement. Then, it seems that it is necessary not only to have resistant colonies but to have a sufficient number of resistant colonies to detect an improvement. ICL resistance of BM CFCs very likely indicates the presence of a reversion mutation in a long-term HSC population. With the exception of two patients, patients with MMC resistant CFCs demonstrated hematologic stabilization and no leukemia. Taking into account the difficulty in obtaining BM samples and considering that 82% of mosaics defined by the DEB chromosome fragility test in peripheral blood are multilineage mosaics; our data suggests the significance of standard chromosomal fragility test in the detection of multilineage mosaic in FA patients. Nevertheless, our observations also reveal for the first time, the importance of performing analyses of MMC resistance in BM CFCs. This evaluation provides information regarding the origin of mosaicism and, importantly, will assist clinicians regarding potential patient evolution and consequently, to provide better and more personalized care to FA patients.

Based on these findings, mosaicism appears to confer protection from the development of severe hematologic complications in FA. This protection was most evident in the multilineage mosaic cohort in which no cases of leukemia or MDS developed among nine patients. In addition to experiencing an older age of hematologic disease onset, mosaic patients are also less likely to require a HSCT. These findings are consistent with a recent retrospective review of published FA mosaic cases in which mosaic patients with blood count normalization, likely from a reversion in long-term

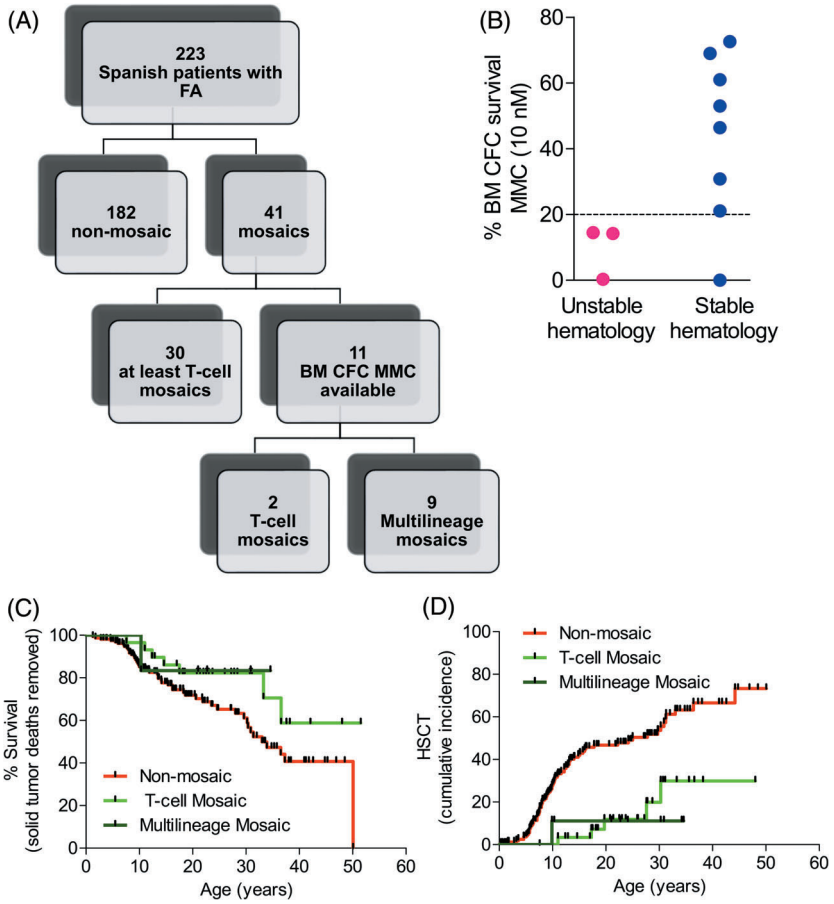

FIGURE 3 Multilineage mosaic patients. (A) Flow diagram indicating the classification of 223 Spanish patients with FA. Forty-one of whom were mosaic, 11 of the mosaic patients had BM CFC MMC available, of whom nine were multilineage mosaic. (B) Higher levels in the percentage of survival of BM CFCs after MMC treatment are associated with a better hematologic evolution. Eleven FA mosaic patients were studied for survival of CFC in BM after MMC treatment (10 nM). Blue or pink circles indicate whether patients had stable or unstable hematology over a sustained time period, respectively. The average follow-up period of these 11 patients is 12 years ranging from 2–30 years. Survival percentages after MMC treatment above 20% in BM CFCs may be indicative of improved hematologic evolution. (C) Overall survival when solid tumors are removed as a cause of death is better in T cells ($p = .072$; hazard ratio 1.79 and 95% CI = 0.95–3.38) or multilineage ($p = .370$; hazard ratio 1.83 and 95% CI = 0.48–6.85) mosaic patients than in non-mosaic patients. No difference was observed between T cell and multilineage mosaic patients ($p = .989$). (D) Higher cumulative incidence of HSCT in non-mosaic vs T cell ($p < .001$; hazard ratio 2.57 and 95% CI = 1.51–4.36) or multilineage ($p = .038$; hazard ratio 2.47 and 95% CI = 1.05–5.80) mosaic patients. No difference was observed between T cell and multilineage mosaic patients ($p = .637$). (A) and (B) curves were compared using a Log-rank (Mantel-Cox) test [Color figure can be viewed at wileyonlinelibrary.com]

primitive HSPCs, were found to have low incidences of BMF or hematologic malignancy.[15] Mosaic patients were similarly found to have relatively limited mortality during the initial two decades of life.[15]

Mosaic patients are more likely to reach adulthood. Not surprisingly, a higher proportion of mosaic patients developed solid tumors likely secondary to the older age of this cohort and increased age-related malignancy risk consistent with the natural history of FA. After removing solid tumors as a cause of death, the long-term survival of mosaic patients is 60%, with the oldest living patient being 52 years old.

Thus, FA mosaicism in hematopoietic cells is a biologic and clinical proof-of principle for autologous gene therapy in FA patients and these results provide a compelling rationale for continued clinical evaluation of autologous gene therapy. Several clinical trials in FA have been conducted in recent years with the latest results indicating that gene-corrected FA HSPCs are capable of engrafting and repopulating over time in the absence of conditioning, due to the selective-advantage over diseased hematopoietic cells, in some instances resulting in meaningful phenotypic correction and hematologic stabilization.[21] In contrast to FA mosaicism in which the reversion mutation likely originates from a single cell, in autologous gene therapy, the degree of genetic and phenotypic correction appears to be dependent on the quantity of gene-corrected cells administered. Longer term follow-up is necessary to further understand clinical outcomes of gene therapy recipients.

In conclusion, our data show that reverse mosaicism is a good prognostic factor in FA. Mosaic patients have later age of onset and less severity of blood disease compared to non-mosaic FA patients and consequently mosaic patients have less requirement for HSCT

and at older ages. Moreover, when solid tumors are removed as a cause of death, mosaic patients have longer survival. Therefore, somatic mosaicism in hematopoietic cells can be considered as a "natural gene therapy" that can be used as a model of favorable evolution for patients treated with gene therapy.

ACKNOWLEDGEMENTS

We would like to thank all participating patients and their families. Surrallés team is supported by the ICREA-Academia program, the Ministerio Español de Ciencia e Innovación (MCI), Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER, "A way to make Europe") (CB06/07/0023, RTI2018-098419-B-I00, Red2018-102 372-T, MCI/AEI/FEDER, UE), the European Commission (P-SPHERE COFUND project), the Fanconi Anemia Research Fund Inc. (FARF), Fundación Ramón Areces (2018) and CIBERER intramural projects. CIBERER is an initiative of the Instituto de Salud Carlos III, Spain. Bueren and Surrallés teams are also supported by FANCOLEN and EUROFANCOLEN projects from the Ministerio Español de Sanidad.

CONFLICT OF INTEREST

Dr. Jordi Surrallés has ongoing research service agreements with Rocket Pharmaceuticals, Inc. Dr. Julián Sevilla receives personal financial compensation for his work as a consultant to Rocket Pharmaceuticals, Inc. Drs. Gayatri Rao, Eileen Nicoletti, and Jonathan D. Schwartz are employees of Rocket Pharmaceuticals, Inc. The remaining authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

María José Ramírez, Roser Pujol, Juan A. Bueren, Jonathan D. Schwartz and Jordi Surrallés planned the study. Roser Pujol, Juan Pablo Trujillo-Quintero, Jordi Minguillón, Massimo Bogliolo, Paula Río, Susana Navarro and José A. Casado performed experiments. Isabel Badell, Estela Carrasco, Judith Balmaña, Albert Català, Julián Sevilla, Cristina Beléndez, Bienvenida Argilés, Mónica López, Cristina Díaz de Heredia provided patient's samples and clinical data. María José Ramírez and Roser Pujol created the database with all the information. María José Ramírez analyzed the data, performed the statistical anlysis and made de figures. María José Ramírez, Eileen Nicoletti, Gayatri Rao, Jonathan D. Schwartz, and Jordi Surrallés wrote the manuscript and all co-authors interpreted and discussed the data and reviewed the manuscript.

ETHICS STATEMENT

All relevant ethical regulations were applied and informed consent was obtained from all participants. This research has been performed in accordance with the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Ramírez MJ, Pujol R, Trujillo-Quintero JP, et al. Natural gene therapy by reverse mosaicism leads to improved hematology in Fanconi anemia patients. *Am J Hematol*. 2021;96:989–999. https://doi.org/10.1002/ajh.26234