Diagnosis of Fanconi anemia in patients with bone marrow failure

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

Patients with bone marrow failure and undiagnosed underlying Fanconi anemia may experience major toxicity if given standard-dose conditioning regimens for hematopoietic stem cell transplant. Due to clinical variability and/or potential emergence of genetic reversion with hematopoietic somatic mosaicism, a straightforward Fanconi anemia diagnosis can be difficult to make, and diagnostic strategies combining different assays in addition to classical breakage tests in blood may be needed.

Design and Methods

We evaluated Fanconi anemia diagnosis on blood lymphocytes and skin fibroblasts from a cohort of 87 bone marrow failure patients (55 children and 32 adults) with no obvious full clinical picture of Fanconi anemia, by performing a combination of chromosomal breakage tests, FANCD2-monoubiquitination assays, a new flow cytometry-based mitomycin C sensitivity test in fibroblasts, and, when Fanconi anemia was diagnosed, complementation group and mutation analyses. The mitomycin C sensitivity test in fibroblasts was validated on control Fanconi anemia and non-Fanconi anemia samples, including other chromosomal instability disorders.

Results

When this diagnosis strategy was applied to the cohort of bone marrow failure patients, 7 Fanconi anemia patients were found (3 children and 4 adults). Classical chromosomal breakage tests in blood detected 4, but analyses on fibroblasts were necessary to diagnose 3 more patients with hematopoietic somatic mosaicism. Importantly, Fanconi anemia was excluded in all the other patients who were fully evaluated.

Conclusions

In this large cohort of patients with bone marrow failure our results confirmed that when any clinical/biological suspicion of Fanconi anemia remains after chromosome breakage tests in blood, based on physical examination, history or inconclusive results, then further evaluation including fibroblast analysis should be made. For that purpose, the flow-based mitomycin C sensitivity test here described proved to be a reliable alternative method to evaluate Fanconi anemia phenotype in fibroblasts. This global strategy allowed early and accurate confirmation or rejection of Fanconi anemia diagnosis with immediate clinical impact for those who underwent hematopoietic stem cell transplant.

Key words: Fanconi anemia, inherited aplastic anemia/bone marrow failure, fibroblasts, somatic mosaicism.

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Introduction

Bone marrow failure syndromes (BMF) are a heterogeneous group of acquired or inherited diseases, which characteristically feature decreased production of hematopoietic cells in the marrow.[1-3] Inherited diseases include Fanconi anemia (FA), dyskeratosis congenita, Shwachman-Diamond syndrome, Diamond-Blackfan anemia and amegakaryocytic thrombocytopenia.[2,3] FA patients often, but not always, present with a combination of various congenital abnormalities (short stature; thumb and radius deformities; microphtalmia and *peculiar* facies; skin hyperpigmentation, such as *café-au-lait spots*; cardiac, renal, genitourinary and/or other malformations).[2,4-8] Most FA patients will develop BMF throughout the course of the disease, usually during their first and second decades of life[9,10] and, for the majority of patients, the suspicion of FA will only be made after the onset of pancytopenia. There is also a strong predisposition to hematologic and epithelial malignancies,[9-13] with cumulative probabilities of an FA patient developing MDS/leukemia being approximately 40% by age 30 years, and a few patients can present with acute leukemia or myelodysplasia at diagnosis.[10] It is crucial, for family counseling and treatment, to identify patients with FA as early as possible. Patients with BMF who happen to have underlying undiagnosed FA will not respond to immunosuppression therapy, which is usually given to treat patients with idiopathic aplastic anemia.[14] Moreover, due to a hypersensitivity to chemotherapy agents, patients with FA will often die of toxicity if given conventional conditioning for HSCT and, therefore, less myeloablative regimens have been used in this population.[15-17] In addition, being at higher risk of developing malignancies, patients with FA will also need appropriate cancer surveillance throughout life.[11-13] Due to the high variety of genes and mutations (13 FA genes have been identified, the most frequently involved being *FANCA, -C, -G* and *-D2*),[18-21] a single genetic test cannot be used as a first approach for FA diagnosis in unselected BMF patients. The biological diagnosis of FA is primarily based on the exquisite sensitivity of peripheral blood lymphocytes (PBL) to DNA interstrand cross-linking chemicals such as diepoxybutane (DEB) or mitomycin C (MMC). The chromosomal breakage test with these agents is the technique of reference for diagnosing FA[22] and, in the vast majority of cases, a precise diagnosis can be made with careful history, physical examination and a positive chromosomal breakage test on PBL. Other tests carried out on PBL include cell cycle analysis[23] and evaluation of FANCD2 monoubiquitination (which can positively diagnose FA-core patients).[24] However, all these tests can be falsely negative in patients who develop hematopoietic reversion and somatic mosaicism. Hematopoietic reversion occurs when, after a spontaneous genetic event in a hematopoietic stem cell (i.e., reverse point mutation or intragenic recombination), one FA allele is corrected, with a consequent recovery of a normal or subnormal protein activity and cellular phenotype.[25,26] Because there has been no evidence that this same phenomenon could happen in primary skin fibroblasts, these cells have been used to overcome false negative results in PBL due to somatic mosaicism.[27-30]

Here, we describe a cohort of 87 patients with BMF and no strong clinical evidence of FA, who were subject to a combination of classic and innovative FA tests on PBL and on primary skin fibroblasts, aiming to reveal unexpected FA cases and rule out this diagnosis in others. Clinical presentation and biological confirmation of 7 FA patients identified are detailed, and strategies for a comprehensive and precise diagnosis of FA in patients presenting with BMF are discussed.

Design and Methods

Patients' characteristics and data collection

From February 2002 to January 2007, 87 consecutive patients were included in this cohort. They were either seen at (n=75) or samples referred to (n=12) Hôpital Saint-Louis, Paris. In both cases, at least one medical appointment with complete history and physical examination were performed by FA-experienced physicians and data recorded. Informed consent was obtained from the patients and/or their relatives. The study was approved by the Review board of the Fédération d'Hématologie, Hôpital Saint-Louis, Paris, France. Patients included in the cohort were children or adults with bone marrow failure (at least one isolated or combined peripheral cytopenias and hypoplastic/aplastic bone marrow aspirates/biopsies), but without a full clinical picture of FA based on commonly seen findings and subjective impression of the evaluating physician. This included BMF patients (i) without any evidence of an associated underlying etiology, (ii) with only an isolated non-specific positive sign in history or physical examination (i.e. isolated short stature, or café-au-lait spots, or history of consanguinity), and (iii) patients with suspected genetic syndromes (based on clinical signs and/or family history), probably other than FA, who were tested to rule out the diagnosis of FA. For all cases, cytopenia was defined as peripheral blood Hb<10.0 g/dL, neutrophils<$\times 10^9$/L and/or platelets<100×10^9/L.[9] Marrow hypoplasia/aplasia was defined based on standard histopathological diagnostic criteria.[31] Patients further identified as having hypoplastic myelodysplasia (MDS) were also analyzed. The cohort included 49 female and 38 male patients (55 children and 32 adults) and, at diagnosis, median age was 15.0 years (range 0.6-50.8), median WBC count was 2.9×10^9/L (range 0.2-9.7), median neutrophil counts 0.7×10^9/L (range 0.02- 5.9), median hemoglobin 8.7 g/ dL (range 2.9-15.9) and median platelet levels were 40×10^9/L (range 1.2-423). Clinical details are given in Table 1, and additional details in the *Online Supplementary Tables S1 and S2*.

Of note, during the period of the study, 51 other patients with an obvious or a previously known diagnosis of FA were firstly evaluated in our center. Considering that the diagnosis was not questionable in these cases, these patients were not included in the present study, the aim of which was to address the question of unexpected FA diagnosis.

Fanconi anemia biological diagnosis

Peripheral blood (in the 87 patients) and skin biopsies (in 64 out of 87) were collected. Fragments of skin were obtained with a minimally invasive 3-mm punch using standard techniques[32] and skin fibroblasts were cultured as previously described.[29]

The following FA tests were used: (i) classic chromosomal breakage test on PBL, (ii) FANCD2 monoubiquitination by Western blot on PBL (in order to evaluate the ability of the FA core complex to monoubiquitinate FANCD2, and the level of expression of the FANCD2 protein), (iii) FANCD2 monoubiquitination by Western Blot on primary skin fibroblasts (in order to overcome the possibility of revertant cases which would give negative PBL tests), (iv) MMC-sensitivity test, flow-cytometry based, on skin fibroblasts (in order to evaluate the possibility of *downstream* FA groups which FANCD2 immunoblot would not detect), and finally, when FA was diagnosed, (v) retroviral FA complementation group and (vi) mutation analysis.

Chromosomal breakages on phytohemaglutinin (PHA)-stimulated PBL, FANCD2 immunoblot on PHA-stimulated PBL and on primary skin fibroblasts were performed as previously described.[29] Two distinct fibroblast lines were grown in separate wells from the same skin biopsy and further tested in most cases, in order to overcome potential *in vitro* FA reversion of fibroblasts (which was not found in FA patients of this study, nor in fibroblasts from a larger FA patient cohort, (D. Chamousset and J. Soulier, unpublished data, 2008).

A new flow-cytometry based, MMC-sensitivity test on fibroblasts, was performed as follows. At Day 0, growing primary fibroblasts were trypsinized, washed, and cells were replated in 24 multi-well plate at 10^5 cells per well in 1/mL RPMI-FCS at 37°C at 5% CO_2. At Day 1, Mitomycin C (MMC, Sigma Aldrich, www.sigmaaldrich.com/) was added at concentrations of 0, 0.5, 1, 2.5, 5, 10, and 25 ng/mL. At Day 4 (72 hours exposure to MMC), cells were washed, trypsinized and harvested (neither permeabilization nor fixation). Propidium iodide (PI, Sigma Aldrich) was added at a final concentration of 10 mg/mL in PBS-FCS, and the fluorescence was immediately analyzed by flow cytometry after gating of the cells by standard two-parameter forward scatter (FSC; size) and side scatter (SSC; granularity), using a FACSCalibur Flow Cytometer and CellQuest analysis system (BD Biosciences, *www.bdbiosciences.com*). The fraction of dying cells, which allows cellular permeabilization and PI uptake,[33] was measured by a shift on FL2. By including healthy and FA control cases in the experiment, comparison of the cell sensitivity to an increasing concentration of MMC clearly discriminates the FA phenotype.

This new flow-based MMC-sensitivity test in fibroblasts was validated by analyzing primary fibroblasts of 34 molecularly-proven mutated FA cases (25 FA-A, 2 FA-G, 1 FA-C, 3 FA-D2, 2 FA-D1/BRCA2, 1 FA-J), including 3 FA patients previously diagnosed with a reversion in blood, and from 10 non-FA control cases including 3 healthy donors having plastic surgery. In all cases, MMC-sensitivity and MMC-resistance, respectively, were as expected (Figure 1). Moreover, primary cells from non-FA chromosome fragility syndromes were tested: Nijmegen syndrome[34] (n=2 cases, both NBS1-mutated), dyskeratosis congenita (n=1, TERT-mutated), Seckel syndrome (n=1), VACTERL syndrome (n=2, non BRCA2-mutated), and xeroderma pigmentosum (n=2, both XPC-mutated). A normal MMC-resistant phenotype was found in all these cases, further demonstrating the specificity of this method to accurately diagnose FA (Figure 1).

Table 1. Clinical characteristics of 87 patients with bone marrow failure.

Median age in years (range)	15.0 (0.6-50.8)
Gender (female:male)	49:38
FBC at enrollment	
Hb (g/dL), median (range)	8.7 (2.9-15.9)
WBC (10^9/L), median (range)	2.9 (0.2-9.7)
Neutrophils ($\times 10^9$/L), median (range)	0.7 (0.02-5.9)
Platelets ($\times 10^9$/L), median (range)	40 (1.2-423)

FBC: full blood counts; Hb: hemoglobin; WBC: white blood cell counts.

In the FA patients identified in the BMF cohort, complementation groups were determined by retroviral transduction as previously described.[24] FANCA and FANCC mutations, and FANCA deletions, were searched for in genomic DNA from FA fibroblasts by direct sequencing and by MLPA analysis (Multiplex ligation-dependent probe amplification, SALSA KIT P031/P032 FANCA, MRC Holland, www.mrc-holland.com), respectively. The Rockefeller FA mutation database (*www.rockefeller.edu/fanconi/mutate/*) was used to analyze the FANC mutations. Somatic reversion was evidenced by comparing genomic DNA from PBL and fibroblasts.

Results

Results of Fanconi anemia tests in blood

Chromosomal breakage tests on PBL were negative (no increase in MMC-induced breaks) in 75/87 cases, positive in 3, ambiguous in 2 cases (significantly more breaks than in normal controls but less than the usual Fanconi range), and in 7 patients metaphases could not be obtained. Results of FANCD2 monoubiquitination by Western Blot on PBL were normal (2-band FANCD2 pattern) in 83/87 cases, and abnormal (single band 'FA core' pattern, i.e. no FANCD2-L isoform) in 4 including the 3 patients with breaks and one patient with MDS and ambiguous chromosomal breakage test (patient H48). In summary, after the biological evaluation of blood samples, 4 FA patients (H11, H19, H48 and H61) were diagnosed in the cohort (see flow-chart in Figure 2). A patient (H04) with ambiguous breaks and a normal FANCD2 pattern remained questionable at this point.

Results of Fanconi anemia tests in fibroblasts, including the new flow-based sensitivity test

Primary fibroblasts could be obtained in 64 patients. Results of FANCD2 tests in fibroblasts were normal in 59/64 cases, and abnormal (no FANCD2-L isoform, FA

core pattern) in 5, including 2 patients (H04 and H38) with hematopoietic reversion who had a normal pattern in PBL (Figures 2 and 3). The fibroblasts were tested using our new flow-based MMC-sensitivity test (after validation of this method using FA and non-FA controls, including other chromosomal instability syndromes, see the Methods section and Figure 1). MMC-sensitivity results in primary fibroblasts in the 64 cases were abnormal (hypersensitivity) in 6 cases, including one downstream patient otherwise undetected (H60), and normal (MMC-resistant) in the remaining 58. No skin sample was available for one additional FA patient who had the diagnosis previously confirmed in blood (H11; breaks and *FA-core* pattern). In summary, after the evaluation of skin fibroblasts, 3 other FA patients with hematopoietic mosaicism were identified in this cohort (H04, H38 and H60), in addition to the 4 above-mentioned who were diagnosed from blood samples (total n=7 FA patients). With this approach, independently of FA subtype and hematopoietic reversion, the MMC-sensitivity on fibroblasts was able to ultimately differentiate a FA from a non-FA patient.

Final diagnoses

After a comprehensive evaluation with thorough clinical evaluation and a combination of FA tests, patients were given a final diagnosis (Table 2).

Seven FA patients were identified in this cohort of 87 patients with BMF, 3 of them presenting with hematopoietic reversion and 4 with atypical presentations, including one patient who was initially thought to have an idiopathic aplastic anemia with no sign of FA in history or physical examination (Table 3). Five of these patients were further assigned to the FA-A group after retroviral complementation group analysis, one to FA-C, and based on the FANCD2 test in fibroblasts, the seventh was considered a *downstream* group. Accordingly, *FANCA* point mutations and/or deletions were identified in 5 patients and *FANCC* mutations in one patient. In addition, reverse mutations were determined by blood versus fibroblast comparative analysis in the 2 FA-

Table 2. Final retained diagnosis for the 87 bone marrow failure patients after clinical and laboratory evaluation.

Fanconi anemia (n=7)	
Without hematopoietic reversion	4
With hematopoietic reversion	3
Idiopathic aplastic anemia (n=52)	
No association	44
PNH clone	3
Viral hepatitis	1
Autoimmune hepatitis	1
Seronegative hepatitis	1
History of drugs (NSAIDs, ethosuximide)	2
Other inherited syndromes (n=28)	
Dyskeratosis congenita	4
Seckel syndrome	1
Blackfan-Diamond anemia	2
Uncategorized (likely inherited syndromes)	21

PNH: paroxysmal nocturnal hemoglobinuria; NSAIDs: non-steroidal anti-inflammatory drugs.

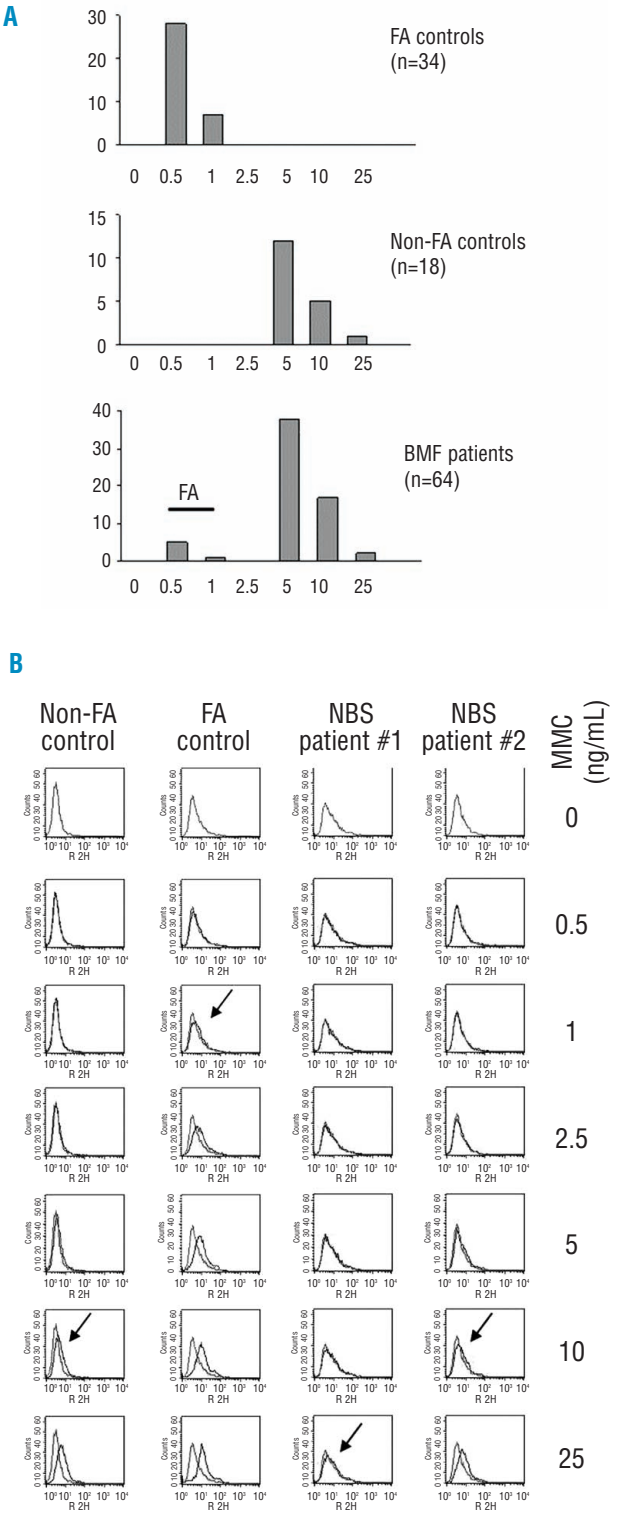


Figure 1. A new flow-based MMC-sensitivity test to diagnose Fanconi anemia (FA) on fibroblasts. (A) Graphs representing the MMC-sensitivity data in FA and non-FA cells: on the X axis are plotted the minimal MMC concentration at which dying cells were detected; Y axis are the number of cases. FA (top panel) and non-FA (middle panel) controls, including other fragility syndromes, and BMF patients (bottom panel) data are shown. (B) MMC-sensitivity flow data from two *NBS1*-mutated Nijmegen patients fibroblasts, compared to a non-FA and a FA-A (EGF125) control patients. FA-A, but not the *NBS1*-mutated fibroblasts, clearly demonstrated greater sensitivity to MMC compared to non-FA control in the same experiment, as detected by PI intracellular uptake.

A patients who presented with somatic mosaicism (Figure 3 and Table 3). Clinical characteristics and biological findings for the 7 FA patients identified in this cohort are shown in Table 3.

FA diagnosis was ruled out in 78 patients (Table 2). For 52 of these, a final diagnosis of idiopathic aplastic anemia was retained, including 13 patients with isolated positive signs in history or physical exam (i.e. isolated short stature, *café-au-lait spots*, or history of consanguinity). Paroxysmal nocturnal hemoglobinuria (PNH) clones, associated acute hepatitis, or medication use were found in very few cases (Table 2). Other patients had likely inherited diagnoses (n=28), including dyskeratosis congenita in 4 (one of them with the severe Hoyeraal-Hreidarsson form), Blackfan-Diamond in 2 (initially systematically evaluated to rule out FA diagnosis), Seckel syndrome in 1, and probable *uncategorized inherited syndromes* in the remaining 21. Characteristics of the BMF presentation, biological results, and the final diagnoses for patients who were likely to have an underlying inherited condition (n=30, 2 FA), and for those with only one isolated positive clinical finding (n=18, 4 FA), are shown in *Online Supplementary Tables S1 and S2*, respectively.

Discussion

Although FA has been known for decades and the main involved genes and proteins have now been described, making a correct and early diagnosis can still be difficult. FA patients who don't present with the association of the most common clinical findings and those, in rare occasions, who have fully negative FA tests in PBL due to hematopoietic reversion can still be undiagnosed and not be offered the best available treatment in a timely manner. This is particularly true for patients who will be treated with HSCT and for whom ruling out a diagnosis of FA is imperative in order to avoid the excess of toxicity with conventional conditioning regimens. Here we evaluated a series of 87 patients with BMF who did not have a clear initial diagnosis of FA (based on history and physical exam) and to whom classical and innovative FA diagnostic tests were offered. The hypothesis was that we would be able to find some FA patients in this population of 'non-typical Fanconi' BMF syndromes and, importantly, to definitely rule out such a diagnosis in others, ultimately opening the discussion about the optimal strategy for FA diagnosis/exclusion in BMF patients. This study focused on BMF patients with questionable diagnosis, a fairly common clinical situation, and therefore patients firstly referred to our center with previously established or obvious diagnosis of FA were excluded from the present analysis (n=51 over the same period of the study), as were those presenting with isolated physical signs or early-onset cancer but without BMF. To perform the evaluation, we used a large panel of FA tests, including functional and molecular analyses on blood and skin fibroblasts. We developed a new flow-cytometry-based MMC-sensitivity assay that we found highly sensitive and specific, including distinguishing FA from other DNA fragility syndromes (Figure 1). Chromosome breakage, FANCD2 test, cell cycle analysis, or growth inhibition tests have been reported in fibroblasts and can also be used to diagnose FA when somatic mosaicism is suspected in blood.[27-30,35] In our cohort of 87 patients with BMF, 7 FA cases were identified, including 3 with hematopoietic reversion. It is possible that this relatively high incidence of FA patients found in this study may be partially inherent to our status of reference center institution, where cases with previously non-established diagnosis tend to be referred to for evaluation. In the 7 FA patients, 3 were children, including 2 who did not present with obvious clinical signs for a FA diagnosis. In fact, patient H04 had one single *café-au-lait* spot as only abnormality (and also had hematopoietic reversion in lymphocytes), and patient H11 did seem to have an idiopathic aplastic anemia without any additional clinical sign. On the other hand,

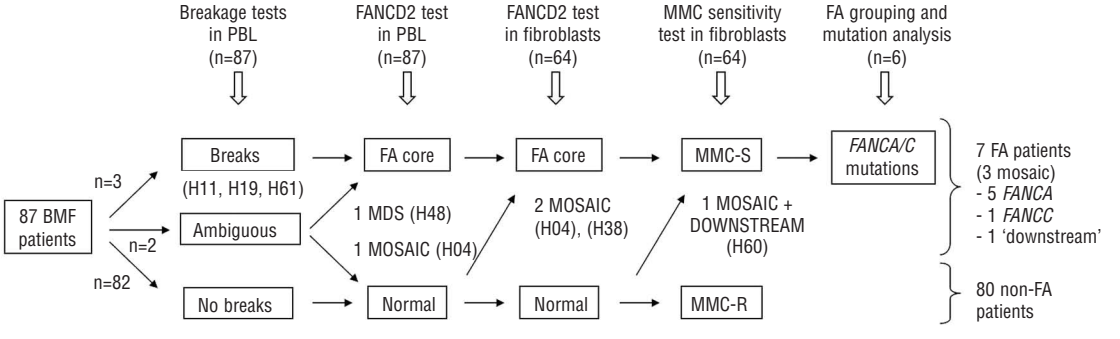


Figure 2. Flow-chart of the Fanconi anemia (FA) work-up in the 87 BMF patients. PBL and primary fibroblasts were tested as indicated. Skin fibroblasts were obtained in 64 patients. For breakage tests, negative (n=75) and failure (n=7, no metaphase obtained) cases were grouped to make the figure simpler, considering that all failure cases further turned out to be non-FA (normal FANCD2 pattern in PBL and normal results in fibroblasts). In the FA patients who were diagnosed, 5 were further grouped as FA-A, one was FA-C, and one was 'downstream' with hematopoietic mosaicism. *FANCA* and *FANC* mutations were identified, and complete *FANCA* reversion was found in one allele in the PBL of the 2 FA-A with somatic mosaicism (Figure 3). Abbreviations: FA core, no FANCD2-L isoform; MMC-S, hypersensitivity to mitomycin C (MMC); MMC-R, no hypersensitivity to MMC.

Table 3. Clinical and biological characteristics of the 7 fanconi anemia patients identified in the cohort.

Patients*	Age (y)	BMF presentation	All positive findings in history or PE	Biological diagnoses
H04, M (EGF056)	10.2	Worsening thrombocytopenia diagnosed at age 9 years	**History**: Guillain-Barré at age 5 years, hypospadias. **PE**: one single *café-au-lait* spot; no short stature, malformation or 'peculiar' facies.	**PBL**: breaks inconclusive and FANCD2 normal. **Fibroblasts**: both FANCD2 and MMC-sensitivity abnormal. **Diagnosis**: FA 'core' with hematopoietic reversion **Complementation group**: FA-A. **Molecular diagnosis**: *FANCA* mutations: c[1115del4] / p[V372A fs X42]; c[3913C>T] / p[L1305F]. **Reversion in PBL**: absence of c[1115del4].
H11, F (EGF003)	12.1	Referred for HSCT with a history of aplastic anemia diagnosed at 9 years, refractory to treatment with ATG/CSA	**History**: no previous hematologic disorders, no consanguinity in the family. PE: normal: no short stature, no malformative syndrome; normal skin and facies.	**PBL**: both breaks and FANCD2 abnormal. **Diagnosis**: FA 'core' **Fibroblasts**: not available. **Molecular diagnosis**: *FANCC* mutations: c[IVS06+1G>A del]; c[1207T>C] / p[W403R]
H19, M (EGF059)	35.9	New onset fatigue	**History**: psychotic disorder diagnosed at age 30 years and treated with neuroleptics; no previous hematologic disorders, no consanguinity in the family; two other healthy siblings. **PE**: short stature (height at -3.3 SD), *peculiar* facies, stellate angiomas; no other malformations or typical *café-au-lait* spots. **Follow-up**: esophageal epidermoid carcinoma diagnosed at the age of 36 years.	**PBL**: both breaks and FANCD2 abnormal. **Diagnosis**: FA 'core' **Fibroblasts**: both FANCD2 and MMC-sensitivity abnormal. **Complementation group**: FA-A. **Molecular diagnosis**: *FANCA* mutation: c[3791A>G] / p[T1131A]
H38, F (EGF071)	47.5	Slowly progressive cytopenia over a period of 10 years	**History**: family history of consanguinity, irregular menses and early menopause at 30 years, vocal cord neoplasia treated by local radiotherapy at 38 years, cured. Repeat normal chromosomal breakage test on PBL. **PE**: borderline short stature (height at -2 SD), some skin hypopigmentation; no malformations, *café-au-lait* spots or *peculiar* facies. **Bone marrow**: hypoplastic MDS.	**PBL** breaks and FANCD2 normal. **Fibroblasts**: both FANCD2 and MMC-sensitivity abnormal. **Diagnosis**: FA 'core' with hematopoietic reversion Complementation group: FA-A. **Molecular diagnosis**: *FANCA* mutations: c[3917delTT] / p[F1306S Fs X6]; c[2172-2173dupG] / p[T724T Fs X70]. **Reversion**: no c[3917delTT] in PBL
H48, M (EGF065)	50.8	New onset fatigue	**History**: surgical correction of unilateral supernumerary thumb in childhood, 5 healthy siblings. **PE**: borderline short stature (-2.1 SD). **Bone marrow**: hypoplastic MDS (1q+, 11q+) with blasts.	**PBL**: breaks inconclusive and FANCD2 abnormal. **Diagnosis**: FA 'core' **Fibroblasts**: both FANCD2 and MMC-sensitivity abnormal. Complementation group: FA-A. **Molecular diagnosis**: *FANCA* mutations: c[2T>C] / p[M1T]; c[3391A>G] / p[T1131A].
H60, F (EGF123)	11.6	BMF on routine PBC	**History**: consanguinity, congenital renal tubulopathy. **PE**: short stature (-4 SD), conjunctival telangectasias, abnormal dentition, facial dysmorphism, microcephaly.	**PBL**: breaks and FANCD2 normal. **Fibroblasts**: FANCD2 normal and MMC-sensitivity abnormal. **Diagnosis**: FA 'unidentified downstream' with hematopoietic reversion.
H61, M (EGF089)	26.2	New onset fatigue	**History**: previously healthy; no positive family history. **PE**: borderline microphtalmia, low-set thumbs, one supernumerary nipple; no 'peculiar facies', skin lesions, short stature or other malformations. Bone marrow: hypoplastic MDS (1q+).	**PBL**: both breaks and FANCD2 abnormal. **Diagnosis**: FA 'core' **Fibroblasts**: both FANCD2 and MMC-sensitivity abnormal. **Complementation group**: FA-A. **Molecular diagnosis**: *FANCA* mutations: deletion exons 11-20; deletion exons 16

Once the diagnosis was made, FA patients were further renamed according to our EGF standard nomenclature (unique reference number). BMF: bone marrow failure; M: male; F: female; PE: physical exam; PBL: peripheral blood lymphocytes; MMC: mitomycin C; FANCD2: immunoblot for detection of FANCD2-L monoubiquitinated isoform; FA-A: FA complementation group A; FANCA: Fanconi anemia group A gene; SD: standard deviation; ATG: anti-thymocyte globulin; CSA: cyclosporine; HSCT: hematopoietic stem cell transplant; MDS: myelodysplastic syndrome.

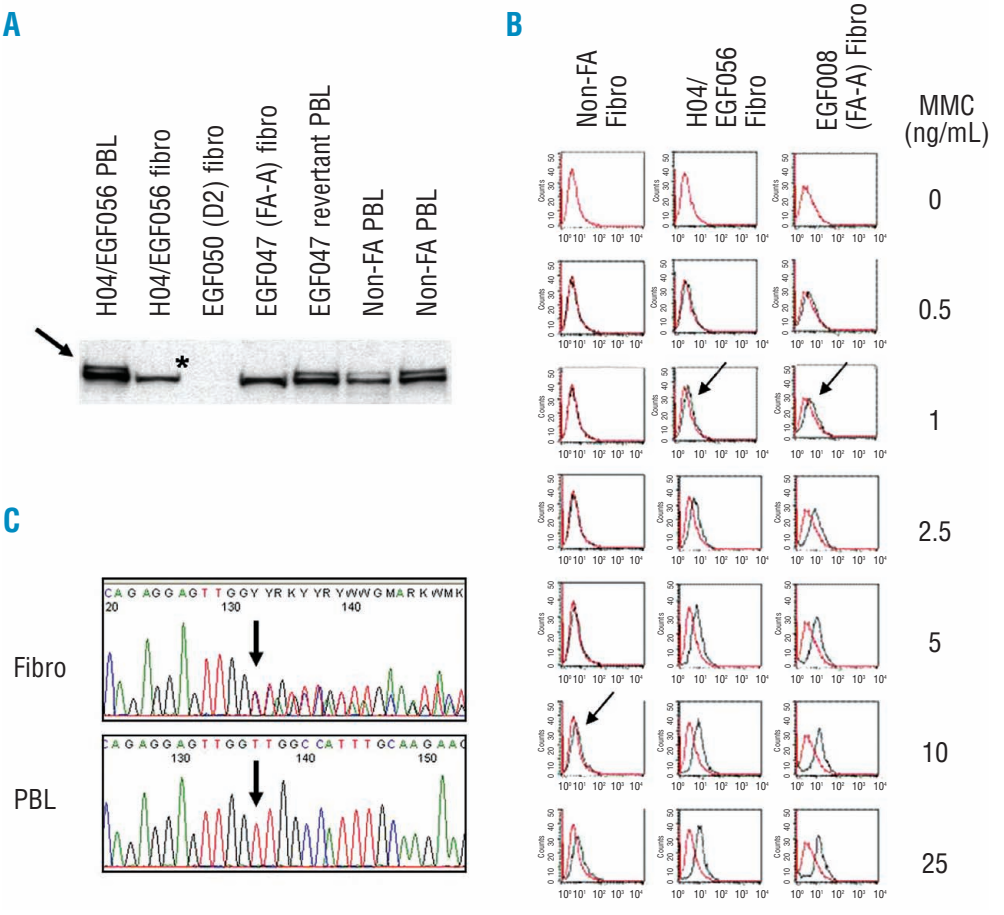
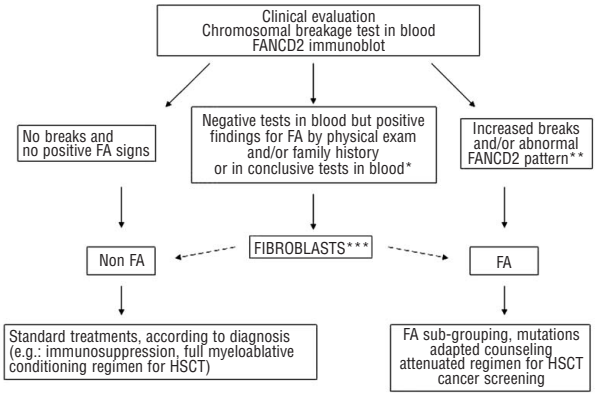


Figure 3. FA diagnosis in patient H04 with hematopoietic somatic mosaicism. (A) Western blot demonstrated an FA core pattern in the primary fibroblasts (no FANCD2-L isoform, indicated by a star) but a normal pattern in PBL (arrow), respectively. Control FA and non-FA samples are also shown, including an FA-D2 sample in line 3 (with no detectable FANCD2 protein; FANCD2 mutations were detected in this patient, *not shown*). (B) MMC sensitivity test demonstrated a clear hypersensitivity in patient H04/EGF056 (line 2) compared to non-FA (line 1) and FA-A EGF008 (line 3) controls. Arrows show dying cells at lower MMC concentration for each patient as detected by PI uptake. (C) Two FANCA mutations were identified in the fibroblast DNA; Mutation c.[1115-1118del4]/p.[V372A fs X42] (exon 13, arrow) was found in the fibroblast gDNA (top panel) but not in the PBL gDNA (lower panel), demonstrating complete somatic mosaicism.

the third child diagnosed with FA in this cohort (H60) presented with multiple malformations and clinical findings resembling an inherited syndrome other than FA (in addition to the BMF and very short stature). Since she displayed a clear MMC hypersensitivity pattern on several experiments and normal *FANCD2* immunoblot test on fibroblasts, a diagnosis of *FA downstream group* was tentatively retained. Four other FA patients were diagnosed in adulthood. Patient H38 did have, retrospectively, findings suggestive of the disease, but even after evaluation in various other hematology services and laboratories in the country, her diagnosis was still delayed for years due to the late age at presentation (47 years) and the repeatedly full negative chromosomal breakage tests on PBL. With our evaluation, a definite FA diagnosis was established in this patient (FA core pattern and MMC-sensitivity in fibroblasts, and two *FANCA* mutations in fibroblast, with complete reversion of one allele in blood, see Table 3). Three other patients (H61, H19, and H48) remained undiagnosed until their 2nd, 3rd and 5th decades of life respectively, due to the scarcity of positive clinical findings in history and physical exam, and long-standing absence of hematologic complications (Table 3). In these cases, the suspicion of FA was only made after the onset of the hematologic disease. It is possible that delayed FA diagnosis to adulthood was related to somatic mosaicism and/or to hypomorphic FANC mutations.[36]

Importantly, this diagnostic strategy ruled out an FA diagnosis in all of the other patients who had skin samples available, including those who had some evidence of an inherited condition associated to the BMF. Twenty-one patients retained a final diagnosis of an 'uncategorized inherited syndrome' based on the multiplicity of physical exam findings associated to BMF, and/or the positive family history, and also on failing to formally fulfill clinical diagnostic criteria for a known phenotype (Table 2 and *Online Supplementary Table S1*). Further evaluation of patients who share similar phenotypes in this group may provide us with links to new syndromes and genes involved in the development of the bone marrow failure. Fifty-two other patients had a final diagnosis of idiopathic aplastic anemia, including 10 who were questionable before our fibroblast evaluation due to isolated signs (Table 2 and *Online Supplementary Table S2*). Seventeen patients in this cohort were further treated with HSCT for the bone marrow failure and, because the diagnosis of FA had been formally excluded after the PBL and fibroblast evaluation we performed, appropriate standard conditioning regimens were given. Sixteen of them are alive and well, with a median follow-up of 18.3 months (range 0.9-43.2). The additional FA patient H11/EGF003, who seemed to have an idiopathic aplastic anemia dur-



* In case metaphases are not obtained the breakage test must be repeated
** FANCD2 test is optional; we find it helpful and we use it systematically. A negative FANCD2 test does not definitely rule out FA because it can be related to somatic mosaicism or downstream FA group.
*** Fibroblasts can be analyzed using cytogenetics techniques (chromosomal breakages), flow cytometry by PI incorporation as described here, or cell cycle analysis.

Figure 4. Suggested diagnostic strategy for evaluating Fanconi anemia in BMF. Initial assessment should include thorough clinical evaluation with detailed personal/family history and physical exam, and chromosomal breakage test in PBL. Patients with no positive FA signs and no increased breaks are considered to be non-FA and are treated accordingly. If breaks are increased, a diagnosis of FA is made and appropriate treatment/follow-up offered. Primary skin fibroblast analyses should be considered in patients where a suspicion of FA remains after negative chromosomal breakage test on PBL (based on positive findings in the clinical assessment), or in patients with inconclusive tests in blood.

ing the initial clinical evaluation but turned out to have positive FA tests, received an adapted dose-reduced conditioning regimen for her HSCT.

Finally, the following questions were raised: (i) should FA screening tests, such as chromosomal breakages on PBL, be performed for all patients with BMF syndromes, including those with a likely diagnosis of idiopathic aplastic anemia, to detect FA cases with atypical presentations? Due to the existence of rare FA patients who present as idiopathic aplastic anemia without any FA clinical signs, i.e. patient H11 in this cohort, it appears that FA should indeed be investigated in all BMF patients, as previously suggested.[2,6] The chromosomal breakage test in blood is effective and sufficient to differentiate FA from idiopathic aplastic anemia without FA clinical signs or familial history. The FANCD2 test is useful to positively diagnose FA, but it fails to detect the very rare downstream FA patients. Fibroblast tests are efficient but demanding (skin biopsy and the results delayed by 4-6 weeks cell growth), so they are not used as first-line screening. (ii) should specific fibroblast analysis be performed when a clinical suspicion of FA remains after negative or inconclusive tests in blood, in order to detect somatic mosaicism or definitely exclude FA? As expected from reported cases of mosaicism in FA,[27-30] we found in this series of 87 BMF patients that the fibroblast analysis was decisive to either confirm or exclude the diagnosis of FA when a suspicion of FA remained after negative or inconclusive tests in PBL. For that purpose, the new flow-based MMC sensitivity test here described proved to be a reliable alternative method to evaluate FA phenotype in fibroblasts. Such patients could then be counseled and treated accordingly, especially when considering immunosuppression therapy, HSCT, and further monitoring of cancer predisposition. Patients with a likely inherited condition other than FA can also be screened for other genetic disorders, and ultimately, new previously uncategorized inherited syndromes associated with BMF may be identified (see patients in *Online Supplementary Table S1*).

Figure 4 summarizes our current proposed diagnostic strategy for evaluating FA in BMF.

In conclusion, a careful and specialized evaluation should be performed in patients where a suspicion of FA remains after initial testing, due to positive history, physical exam findings, and/or inconclusive chromosome tests in blood. Such evaluation should be done in reference centers where a complete set of tests, including fibroblasts analyses (when necessary), appropriate FA and non-FA controls, and mutation analyses are available, with immediate clinical impact for patients with BMF who need an HSCT.

Authorship and Disclosures

FOP: study design, gathering/analysis of clinical data and writing of the paper; DC, GLR, BC, JL, JPV: biological experiments; DS-L: mutation analysis; TL, BB, AB: study design and patient clinical care/follow-up; GS, EG: study design, patient clinical care/follow-up and writing of the paper; JS: study design, gathering/analysis of data, and writing the paper. Thanks to Helen Walden for proofreading the manuscript.

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