

Chromosome fragility in patients with Fanconi anaemia: diagnostic implications and clinical impact

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ABSTRACT

Background Fanconi anaemia (FA) is a rare syndrome characterized by bone marrow failure, malformations and cancer predisposition. Chromosome fragility induced by DNA interstrand crosslink (ICL)-inducing agents such as diepoxybutane (DEB) or mitomycin C (MMC) is the 'gold standard' test for the diagnosis of FA.

Objective To study the variability, the diagnostic implications and the clinical impact of chromosome fragility in FA.

Methods Data are presented from 198 DEB-induced chromosome fragility tests in patients with and without FA where information on genetic subtype, cell sensitivity to MMC and clinical data were available.

Results This large series allowed quantification of the variability and the level of overlap in ICL sensitivity among patients with FA and the normal population. A new chromosome fragility index is proposed that provides a cut-off diagnostic level to unambiguously distinguish patients with FA, including mosaics, from non-FA individuals. Spontaneous chromosome fragility and its correlation with DEB-induced fragility was also analysed, indicating that although both variables are correlated, 54% of patients with FA do not have spontaneous fragility. The data reveal a correlation between malformations and sensitivity to ICL-inducing agents. This correlation was also statistically significant when the analysis was restricted to patients from the FA-A complementation group. Finally, chromosome fragility does not correlate with the age of onset of haematological disease.

Conclusions This study proposes a new chromosome fragility index and suggests that genome instability during embryo development may be related to malformations in FA, while DEB-induced chromosome breaks in T cells have no prognostic value for the haematological disease.

INTRODUCTION

Fanconi anaemia (FA) is a rare genetic disease characterised by bone marrow failure (BMF), congenital malformations, endocrine dysfunctions and cancer predisposition. It was first described in 1927 by the Swiss paediatrician Guido Fanconi¹ and its estimated incidence is about 1–5 cases in one million births in the overall population,² or <1 in 20 000 in some consanguineous ethnic groups.^{3–5}

FA is a genetically heterogeneous disease, as 13 different FA complementation groups and corresponding genes have been currently identified (*FANC-A*, *-B*, *-C*, *-D1/BRCA2*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J/BRIP1*, *-L*, *-M* and *-N/PALB2*).^{6–8} In the USA, with 681 patients with FA subtyped, FA-A is the most common complementation group representing 60.5% of the patients; FA-C and FA-G are also common, accounting for 16% and 10% of the patients respectively, while the other groups are rare.^{2 9 10} The FA-A subtype is, however, over-represented in some geographical regions, including Mediterranean countries such as Spain, with four out of five patients with FA belonging to this complementation group.¹¹

At a molecular level, FA proteins are known to function on a common DNA repair pathway, the FA/BRCA pathway, which is focused on the processing of stalled replication forks generated either spontaneously or in response to drug-induced DNA interstrand cross-links (ICLs) and other types of DNA damage.¹² Genomic instability is, therefore, a hallmark of FA cells. It was first observed in 1966, when Schroeder described a high frequency of spontaneously formed chromosome breaks in cells of patients with FA.¹³ Later, this genomic instability was seen to be highly induced by ICL-inducing agents, such as diepoxybutane (DEB), mitomycin C (MMC) or cis-platin,^{14 15} leading to the development of the first diagnostic test for FA.^{16 17} Although high sensitivity to ICL-inducing agents is the hallmark of FA cells, an accurate diagnosis is compromised in some cases, especially among mosaic patients, who represent 15–25% of all patients.^{18–20} Somatic mosaicism is produced when one of the pathogenic mutations is reverted in a haematopoietic precursor cell. Owing to an increased proliferative advantage, reverted cells can clonally expand and improve a patient's blood counts. Depending on the stage of differentiation of the cell in which the gene correction occurred, reversion may affect all haematopoietic cell lineages, leading to a 'natural gene therapy'.^{20–22} Alternatively, the reversion may affect only some haematopoietic lineages and therefore, it may not lead to an improvement of a patient's haematological condition. As the diagnostic test of chromosome fragility is usually performed on peripheral blood T cells, a high proportion of reverted T cells

can lead to a false negative result. On the contrary, some non-FA individuals can have a number of T cells with chromosome breaks after DEB or MMC treatment and this can be interpreted as mosaicism by non-experienced laboratories, leading to false positives. This is due to overlapping values of currently used chromosome fragility indexes (percentage of cells with breaks or average number of breaks per cell) between non-FA and FA mosaics.⁹

The primary cause of death among patients with FA is BMF, which typically occurs during the first decade of life.²³ While novel therapeutic strategies to cure BMF are under intensive research, including gene therapy and regenerative medicine based on induced pluripotent stem cells,^{24 25} the only currently available curative treatment is haematopoietic stem cell transplant from a compatible donor, which requires previous conditioning of the patient. Regular conditioning regimens, consisting of chemotherapy and radiation, are highly toxic for patients with FA, so milder conditioning regimens must be used.²⁶ In this context, a clear and unambiguous diagnosis is, therefore, essential for the survival of the patients after haematopoietic stem cell transplant.

To further examine chromosome fragility in FA and its diagnostic and clinical implications, we have performed a total of 198 DEB-induced chromosome fragility tests in non-FA individuals and patients with FA where information on subtype, cell sensitivity to MMC and clinical data were available. This large series allowed us to quantify the existing variability in ICL sensitivity among patients with FA and a normal population and, therefore, we propose a new chromosome fragility index that provides a clear cut-off diagnostic level to unambiguously distinguish patients with FA (including mosaics) from non-FA individuals. Spontaneous chromosome fragility and its correlation with DEB-induced fragility is also analysed and discussed. Finally, the relationship between cell sensitivity to ICLs and the patient's clinical severity markers is evaluated, revealing a direct correlation with congenital malformations, but not with the age of onset of haematological disease.

MATERIALS AND METHODS

Patients and samples

Blood samples from patients with clinical suspicion of FA and controls were collected for chromosome fragility evaluation. Clinical data from patients with FA were obtained from their clinicians, including age of onset of haematological disease and the number of congenital malformations. The number of congenital malformations was recorded as skeletal, head, gastrointestinal, cardiac, genitourinary system malformations and mental retardation. Thus, the number of congenital malformations ranged from 0 to 10. This study was approved by the Universitat Autònoma de Barcelona Ethical Committee for Human Research and informed consent was obtained according to the Declaration of Helsinki.

Chromosome fragility test

Chromosome fragility tests on peripheral blood lymphocytes were performed basically as described by Auerbach,¹⁷ with some minor modifications. Three blood cultures were prepared for each patient, including 0.5 ml of blood in heparin and 4.5 ml of culture consisting of 15% fetal bovine serum, 1% antibiotics, 1% L-glutamine and 1% phytohaemagglutinin in RPMI (all reagents from Gibco, Carlsbad, CA, USA). Twenty four hours after culture set-up, two cultures were treated with DEB at a final concentration of 0.1 µg/ml (Sigma, Cat. No 202533, St. Louis,

MO, USA), and the remaining culture was left untreated for spontaneous chromosome fragility evaluation. Forty-six hours after DEB treatment, colcemid was added at a final concentration of 0.1 µg/ml. Cultures were harvested 2 h later when metaphase spreads were obtained according to standard cytogenetic methods and finally, stained with Giemsa. For chromosome fragility evaluation, 25–50 metaphases with 46 (1) centromeres were analysed for each culture. The microscopic analysis was performed with a Leitz Aristoplan microscope and, later with a Zeiss Imager M1 microscope coupled to a computer-assisted metaphase finder (Metasystems, Altlußheim, Germany). The main criteria for the determination of chromosome fragility were as follows: gaps were not counted as chromosome breaks and figures were converted to the minimum number of breaks necessary to form each figure. DEB stock was routinely replaced every 6 months. Before using a new lot, a control fragility assay was performed using an FA lymphoblastoid cell line to ensure that there was no significant variation between lots.

Analysis of cell survival to MMC

The survival of T cells in a patients with FA after MMC treatment was calculated from data obtained during the subtyping studies described in a previous report.¹¹ Briefly, mononuclear cells from peripheral blood were stimulated in plates coated with anti-human CD3 (OKT3 Ortho Biotech, Raritan, NJ, USA) and anti-CD28 (CD28 Pharmingen, San Diego, California, USA) monoclonal antibodies. Five days later, the proliferating T cells were collected and exposed to increasing concentrations of MMC (0–1000 nmol/l) during an additional 5 days in the presence of interleukin 2 (100 U/ml, Proleukin, Chiron Corp, California, USA). Finally, the cells were re-suspended in phosphate-buffered saline–bovine serum albumin (0.05%) containing 0.5 µg/ml propidium iodide (Sigma) and incubated for 10 min at 4°C. Cell viability was determined by flow cytometry based on the propidium iodide exclusion test. In our previous study we observed that 33 nmol/l is the concentration of MMC that best discriminates between MMC-resistant and MMC-sensitive T cells.¹¹ Therefore, survival after exposure to this concentration of MMC was routinely used to discriminate between T cells with a differential response to MMC.

Statistics

Correlations between variables were analysed using Pearson's test when both variables were normally distributed, or Spearman's test if not. To compare means between several groups, analysis of variance and Tamhane for post hoc analysis were used. All statistical analyses were performed using SPSS software package v.16.

RESULTS

DEB-induced chromosome fragility: discriminating between non-FA, FA mosaics and FA non-mosaic patients

Chromosome fragility was evaluated in 105 non-FA individuals and 93 patients with FA using exactly the same conditions. Of the 93 patients included in this analysis, seven were of unknown complementation group and 86 were successfully subtyped. Of these, 70 patients with FA were found to belong to complementation group FA-A, while other complementation groups were rare. A summary of the results obtained on spontaneous and DEB-induced fragility is provided in table 1. Chromosome fragility is usually reported as 'breaks/cell' and 'percentage of aberrant cells'. As shown in figure 1A,B, the number of 'breaks/

Table 1 Spontaneous and diepoxybutane (DEB)-induced chromosome fragility test: summary of results for Fanconi anaemia (FA) and non-FA groups

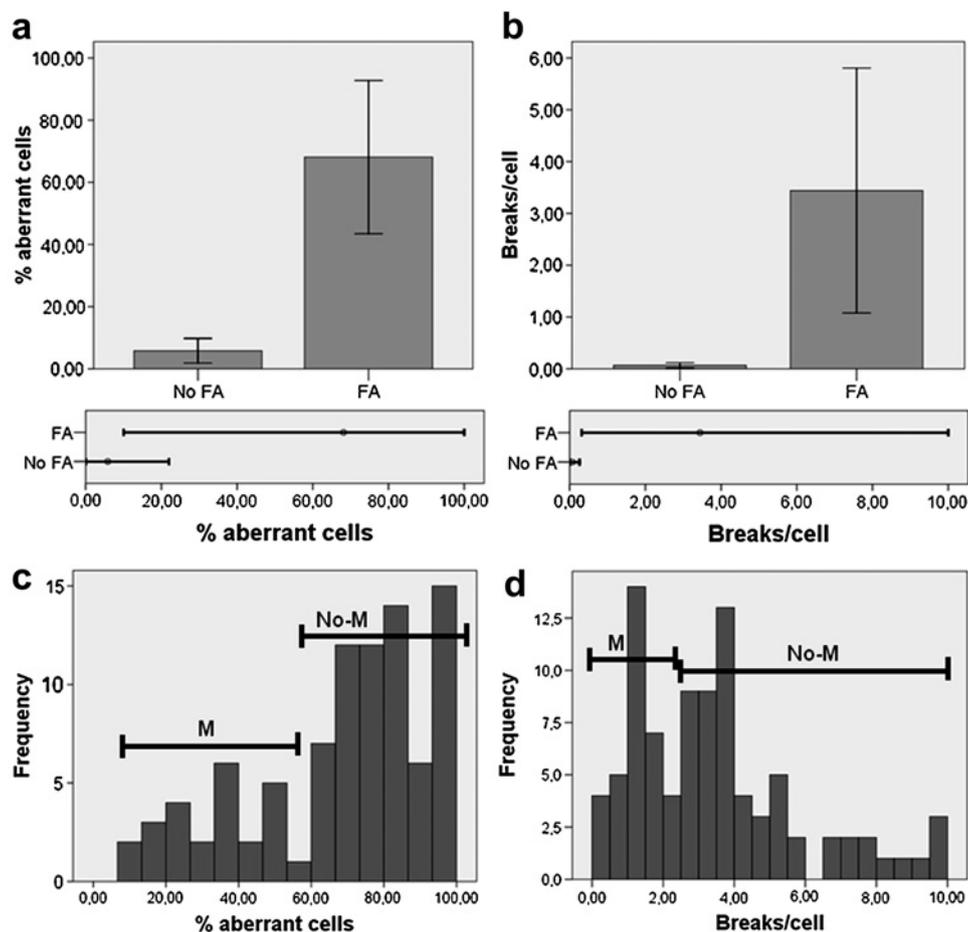
Group	% Aberrant cells			% Multiaberrant cells			Breaks/cell			Breaks/multiaberrant cell		
	n	Mean	Interval	n	Mean	Interval	n	Mean	Interval	n	Mean	Interval
Spontaneous chromosome fragility												
No FA	105	3.21	0–16	105	0.51	0–12	105	0.03	0–0.24	9	2	2.00–2.00
FA	93	15.24	0–64	93	4.57	0–56	93	0.21	0–1.6	51	2.26	2.00–4.00
FA non-mosaic	68	17.56	0–64	68	5.34	0–56	68	0.25	0–1.60	40	2.27	2.00–4.00
FA possible mosaic	8	12.50	0–20	8	3.00	0–8	8	0.17	0–0.28	4	2.50	2.00–3.00
FA mosaic	17	7.23	0–24	17	2.23	0–12	17	0.09	0–0.28	7	2	2.00–2.00
DEB-induced chromosome fragility												
No FA	105	5.83	0–22	105	0.84	0–6	105	0.06	0–0.26	38	2.06	2.00–2.80
FA	91	68.10	10–100	91	54.81	6–100	91	3.44	0.31–10.00	91	5.65	2.40–11.80
FA non-mosaic	66	81.15	60–100	66	67.71	36–100	66	4.34	1.38–10.00	66	5.94	2.80–11.80
FA possible mosaic	8	50.75	46–58	8	32.00	28–38	8	1.38	1.06–1.78	8	3.76	3.16–4.76
FA mosaic	17	25.62	10–38	17	15.44	6–30	17	0.92	0.31–1.52	17	5.43	2.40–9.37

Patients with FA are subdivided in three categories: FA non-mosaic, FA possible mosaic and FA mosaic.

cell' is more than 10 times higher in the FA population than in the non-FA population, while the 'percentage of aberrant cells' is increased 60 times in the FA group. However, the level of variability between patients with FA is very high (see panels below figure 1A,B) and, in some cases, chromosome fragility values in patients with FA overlap those found in non-FA patients. Part of this high variability in the patients with FA is due to the existence of a subgroup of patients with FA who have lower values of 'percentage of aberrant cells' and 'breaks/cell' (figure 1C,D). This subgroup corresponds to patients with FA with a T-cell mosaicism. In this study, patients with FA with <40% of aberrant cells are considered mosaic, while those with $\geq 60\%$ of cells are considered non-mosaic patients with FA. Patients with FA

with a proportion of aberrant cells between 40% and 60% are considered possible mosaics, while waiting for additional evidence of mosaicism. This distinction is based on parallel data on cell sensitivity to MMC, mutational data (pathogenic mutations were identified in 12 out of 17 mosaics), repeated DEB tests over time, ICL sensitivity of all primary fibroblasts available from mosaic patients, and the reverted nature of seven out of eight lymphoblastoid cell lines generated from mosaic patients with FA. All mosaic patients could be subtyped by retroviral complementation, either in blood lymphocytes, when they were sufficiently sensitive to MMC or in fibroblasts, when they were resistant. In the latter case, five out of five established fibroblast cell lines showed the characteristic, MMC-induced

Figure 1 Diepoxybutane-induced chromosome fragility in Fanconi anaemia (FA) and non-FA groups expressed by 'percentage aberrant cells' (A) and 'breaks/cell' (B). Upper panels indicate mean \pm SD and lower panels indicate range. Distribution of patients with FA for 'percentage aberrant cells' (C) and 'breaks/cell' (D). 'M', mosaic; 'No-M', non-mosaic.



G2/M phase cell cycle arrest and, therefore, could be subtyped by retroviral complementation, confirming the mosaic nature of the patients (see below and data not shown).

Discrimination between FA mosaic patients and non-FA is the principal difficulty found when making an FA diagnosis. As shown in figure 2A, the mean level of 'breaks/cell' in FA mosaics is close to that observed for the non-FA population. However, when considering only 'breaks/multiaberrant cell' (breaks observed in cells with two or more breaks) (figure 2B), chro-

mosome fragility level of mosaic patients is equivalent to that seen in FA non-mosaics, even if a few cells with two or more breaks can be found in non-FA individuals (figure 2B, lower panel). Therefore, neither 'percentage aberrant cells' nor 'breaks/cells' or 'breaks/multiaberrant cell' indexes alone are enough to fully discriminate between non-FA and FA mosaic patients. However, when combining 'percentage of aberrant cells' and 'breaks/cell' or 'breaks/multiaberrant cell' in the same graph (figure 2C,D, respectively), a better separation between patient

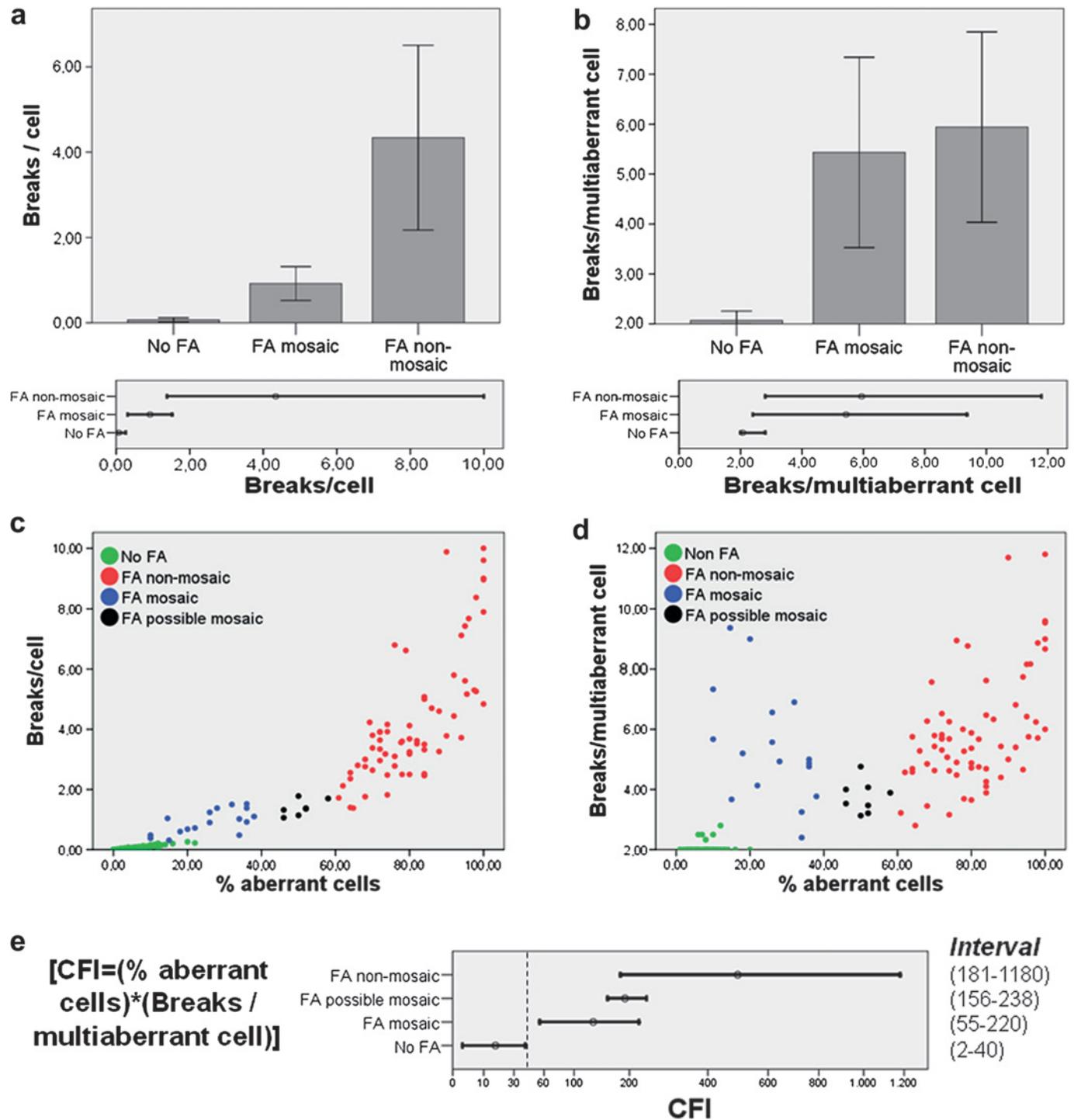


Figure 2 Diepoxybutane-induced chromosome fragility in no-Fanconi anaemia (no FA), FA mosaic and FA non-mosaic groups expressed by 'breaks/cell' (A) and breaks/multiaberrant cell (B). Upper panels indicate mean \pm SD and lower panels indicate range. Two-dimensional distribution of individuals analysed for 'percentage aberrant cells' and 'breaks/cell' (C) or 'breaks/multiaberrant cell' (D). Calculation of Chromosome Fragility Index (CFI) for each group (E). Discontinuous line indicates the threshold (CFI=40).

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subgroups is obtained. Thus, the data graphically presented in figure 2D were transformed into a new index that we called the 'Chromosome Fragility Index' (CFI), obtained by multiplying the other two indexes. As shown in figure 2E, CFI allowed a cut-off value to be established which clearly distinguished the non-FA from the FA population, including mosaics, without overlapping. The results of our study showed that non-FA patients have CFI values <40 , while all patients with FA, including mosaics, have a CFI >55 .

The presence of tri- or tetra-radial figures is a characteristic feature of FA cells upon DEB treatment. In FA individuals, 1373 multiradial figures were found in a total of 4011 metaphases studied. Among all non-FA patients, 5250 metaphases were studied and three cells with one multiradial figure were found in three different individuals (frequency of 0.057% or 1 in 1750 metaphases). Therefore, even though patients with FA have a 600-fold increase in radial figures upon DEB treatment, the presence of figures among the non-FA population is rare, but possible, and should not be interpreted as a positive diagnosis of FA mosaicism.

Spontaneous chromosome fragility

Spontaneous chromosome fragility was also analysed. Results obtained are described in table 1. Overall, higher spontaneous chromosome fragility is seen in patients with FA (figure 3A,B): 5.2-fold in 'percentage aberrant cells' and 8.3-fold in 'breaks/cell', when compared with non-FA patients. However, the variability in spontaneous chromosome fragility is very high in all three groups, and no statistically significant differences could be detected between the non-FA and FA-mosaic groups. As shown in figure 3A,B lower panels, spontaneous chromosome fragility interval values overlap in all three populations. Quantification of the proportion of patients with FA (excluding mosaics) that present a level of spontaneous chromosome fragility in the range of the non-FA population (figure 3C), revealed that 54.4% of patients with FA had spontaneous chromosome fragility within the normal range. In this graph, spontaneous chromosome fragility is measured by integrating 'breaks/cell' and 'percentage aberrant cells' in a 'Spontaneous Chromosome Fragility Index' (SCFI), which results from multiplying 'percentage of aberrant cells' and 'breaks/cell'.

Finally, to further understand the mechanism that produces spontaneous chromosome fragility in patients with FA, the correlation between spontaneous and DEB-induced chromosome fragility was evaluated. As shown in figure 4, a highly significant correlation between spontaneous and DEB-induced chromosome fragility was detected among patients with FA (excluding mosaics) when using 'percentage of aberrant cells' (figure 4A) or 'breaks/cell' (figure 4B).

Cell viability to MMC

To further support the diagnosis by chromosome fragility assay, cell viability upon MMC treatment was also evaluated in non-FA and FA individuals during the genetic subtyping by retroviral-mediated complementation.¹¹ As shown in figure 5A, the non-FA population was resistant to MMC while FA non-mosaics were highly sensitive, as expected. However, a wide range of MMC sensitivity was found among FA mosaic patients. Cell viability to MMC was seen to correlate with the proportion of aberrant cells detected with the DEB-induced chromosome fragility assay among FA mosaic patients (figure 5B). While FA mosaic patients with 20–40% of aberrant cells can be either sensitive or resistant to MMC, patients with $\leq 20\%$ aberrant cells invariably show resistance to MMC (figure 5B). Finally,

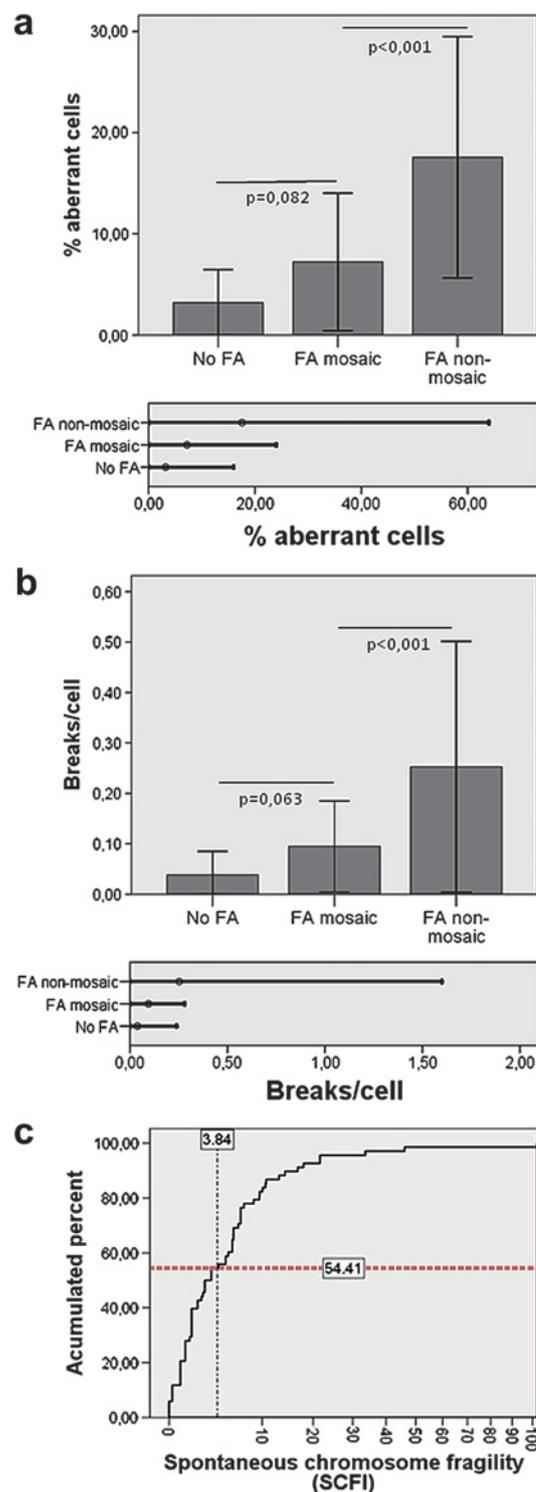
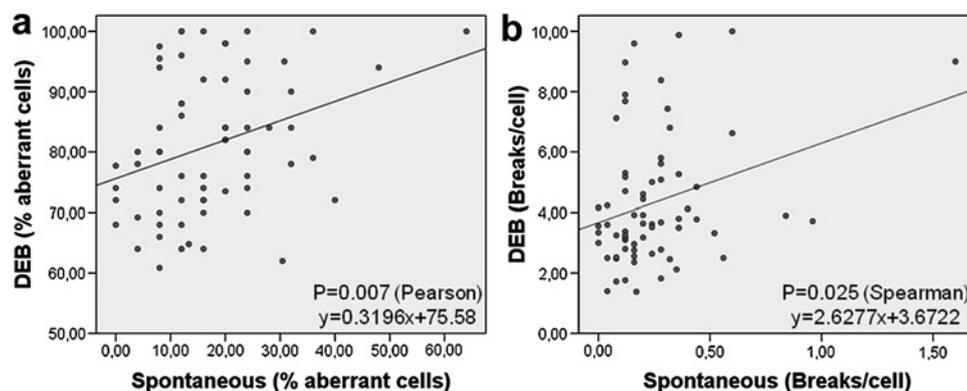


Figure 3 Spontaneous chromosome fragility in no-Fanconi anaemia (no FA), FA mosaic and FA non-mosaic groups expressed by 'percentage aberrant cells' (A) and 'breaks/cell' (B). Upper panels indicate mean \pm SD and lower panels indicate range. Statistical significance is shown. Accumulated percentage of patients with FA (mosaics excluded) versus the Spontaneous Chromosome Fragility Index (SCFI) (C). Reference line on the x-axis indicates the upper level observed in the non-FA population. Reference line on the y-axis indicates the proportion of patients with FA with SCFI below this limit.

a very good correlation between cell sensitivity to MMC and DEB-induced chromosome fragility was also observed in the FA non-mosaic patient group (figure 5C), indicating that the excess

Figure 4 Correlation between spontaneous chromosome fragility and diepoxybutane (DEB)-induced fragility expressed by percentage aberrant cells' (A) and 'breaks/cell' (B).



cell mortality in FA is mainly attributable to the death of cells bearing chromosome breaks.

Correlation between cell sensitivity and severity of patient's clinical phenotype

We finally tried to identify whether ICL sensitivity might be used as a clinical marker to predict the severity of the disease and, therefore, as a prognostic variable at the time of diagnosis. To evaluate the clinical severity, two different clinical markers were used: the number of congenital malformations and the age of onset of haematological disease. Mean age of onset of haematological disease for this population was 6.75 years, similar to that of other previously published cohorts. Correlations with spontaneous chromosome fragility and cell sensitivities to DEB and MMC are presented in table 2. We could not detect any correlation between spontaneous chromosome fragility and clinical markers. However, a good correlation was found between number of congenital malformations and DEB-induced chromosome fragility or cell sensitivity to MMC. As shown in figure 6A,B, cells from patients with FA with a higher number of malformations statistically have more DEB-induced chromosome breaks ($p < 0.001$) and more sensitivity to MMC. To rule out a possible effect of subtype as a confounding factor in this analysis, the same correlation was assessed within the group of FA-A patients with available clinical data ($n=46$), and the same highly significant correlation was detected between the number of malformations and DEB-induced fragility ($p < 0.001$). On the contrary, cell sensitivity to ICLs did not correlate with the age of onset of haematological disease and, therefore, this variable has no prognostic value in FA.

DISCUSSION

The chromosome fragility assay upon treatment with ICL-inducing agents is the most widely used test for the diagnosis of FA, although it is laborious and requires specialised personnel. Among the different ICL inducers, DEB is commonly chosen, owing to high compound stability and high specificity, as no other group of individuals with sensitivity to DEB comparable to that of patients with FA has been described. To further improve our understanding of chromosome fragility in FA and find rational criteria to correctly and unambiguously diagnose patients with FA, including mosaics with a high percentage of reverted cells in their blood, we have performed a comprehensive study to quantify the variability in spontaneous and DEB-induced chromosome fragility among patients with FA and the non-FA population. The results of the chromosome fragility test presented here have been obtained in an ethnically homogenous population of Spanish Caucasian individuals, and the cyto-

netic analysis has been performed systematically in the same laboratory and under controlled conditions over a period of 11 years (1999–2009).

Typically, reported indexes to measure chromosome fragility ('breaks/cell' or 'percentage aberrant cells') allow clear discrimination between the non-FA population and non-mosaic FA patients.¹⁷ However, mosaic patients present intermediate values and can be easily misdiagnosed. Similarly, non-FA individuals with higher sensitivity to ICL due to genetic background or other unknown factors can have a spontaneous or DEB-induced frequency of cells with breaks of up to 16% or 22%, respectively, after DEB. These levels of chromosome damage, together with the presence of multiradial figures in a few cases (we detected at least three non-FA patients with one multiradial figure), can lead to a false-positive diagnosis of mosaicism in a non-specialised laboratory. However, cells with breaks in FA mosaics are usually multiaberrant. This fact led us to develop a novel index (CFI) that integrates 'percentage of aberrant cells' and 'breaks/multiaberrant cell' to unambiguously discriminate mosaic patients from non-FA population, as it takes into account the parameters that are more significant for a correct diagnosis of FA. In our hands a CFI=40 can be used as a cut-off point to separate patients with FA (including mosaics) from the non-FA population. Although this cut-off level may vary between laboratories, we highly recommend implementation of the CFI in diagnostic laboratories as it adds to the integrated analysis of cell distribution of chromosome fragility, resulting in a better and more reliable diagnosis of FA.

FA mosaic patients are usually discriminated from FA non-mosaics according to the percentage of aberrant cells. However, the mosaicism phenomenon is progressive and, therefore, the differentiation between FA mosaic and FA non-mosaic (full FA) patients is not always possible. When restrictive criteria are used—for example, when only patients with FA with $\leq 40\%$ of aberrant cells are considered as mosaics, 18% of Spanish patients with FA are mosaic. With a less conservative upper limit of 50% of aberrant cells, the incidence of mosaicism would increase to $>20\%$. Similar criteria for the detection of mosaicism have been proposed for other specialised laboratories⁹ and our frequency of mosaicism is also within the range previously reported in other populations (15–25%).^{18–20}

Cell viability after MMC treatment can be useful in the discrimination of mosaic patients, as the majority of them are resistant to MMC, given the high proportion of wild-type T cells in their blood. However, some mosaic patients have a high sensitivity to MMC while having a high percentage of cells without breaks in their peripheral blood. The reason for this apparent contradiction is not known. It is also important to mention that the mosaicism observed in the T-cell lineage does

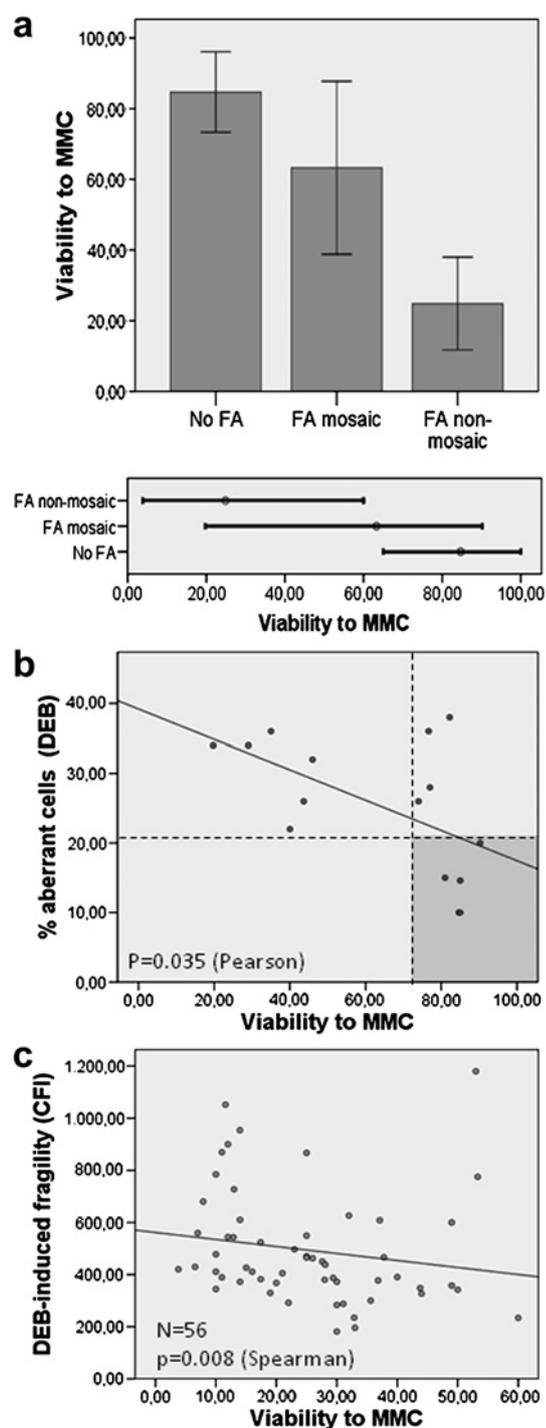


Figure 5 Cell viability to 33 nM of mitomycin C (MMC) in no-Fanconi anaemia (no FA), FA mosaic and FA non-mosaic groups (A). Upper panel indicate mean \pm SD and lower panel indicate range. Correlation between diepoxybutane (DEB)-induced percentage aberrant cells' and viability to MMC in mosaic patients; shaded square indicates mosaic patients with <20% of aberrant cells that invariably show resistance to MMC (B). Correlation between DEB-induced fragility (Chromosome Fragility Index (CFI)) and viability to MMC in FA non-mosaic patients (C).

not always imply normal patient blood counts, and therefore, mosaicism may not necessarily have clinical implications. Likewise we have detected a patient with improved blood counts over time since first being diagnosed (platelets, haemoglobin and neutrophils) but with a stable percentage of aberrant T cells over a period of at least 7 years: DEB tests performed in 2002, 2008 and 2009 with percentages of aberrant cells of 64%, 59% and

Table 2 Correlation between cell sensitivity markers and patient's clinical severity

	Spontaneous fragility (SCFI)		DEB-induced fragility (CFI)		Viability to MMC	
	n	p Value	n	p Value	n	p Value
Number of malformations	54	0.970	52	<0.001	53	0.029
Onset of haematological disease	49	0.935	48	0.711	49	0.988

Number of patients included (n) and statistical significance (p) are shown. CFI, Chromosome Fragility Index; DEB, diepoxybutane; MMC, mitomycin C.

69%, respectively. This observation suggests a lack of mosaicism in the lymphoid lineage of the haematopoiesis, but somatic reversion in the myeloid lineage.

FA has long been considered a spontaneous chromosome fragility syndrome since the pioneering work of Professor Traute Schroeder in 1964.¹³ Results obtained in this study show that while on average the FA population has an increased level of spontaneous DNA damage, 54% of patients with FA (excluding mosaics) have a spontaneous chromosome fragility level within the normal range of non-FA patients. Therefore, spontaneous chromosome fragility, although helpful when positive, cannot be used as a diagnostic tool for FA.

It is well known that patients with FA are highly sensitive to ICLs, although it has been proposed that other types of DNA damage, like oxidative stress, can be responsible for spontaneous chromosome fragility.^{27–29} In this study we show that spontaneous and DEB-induced fragility, as well as cellular sensitivity to MMC are all highly variable among patients with FA, but a good correlation between sensitivity to ICL-inducing agents and

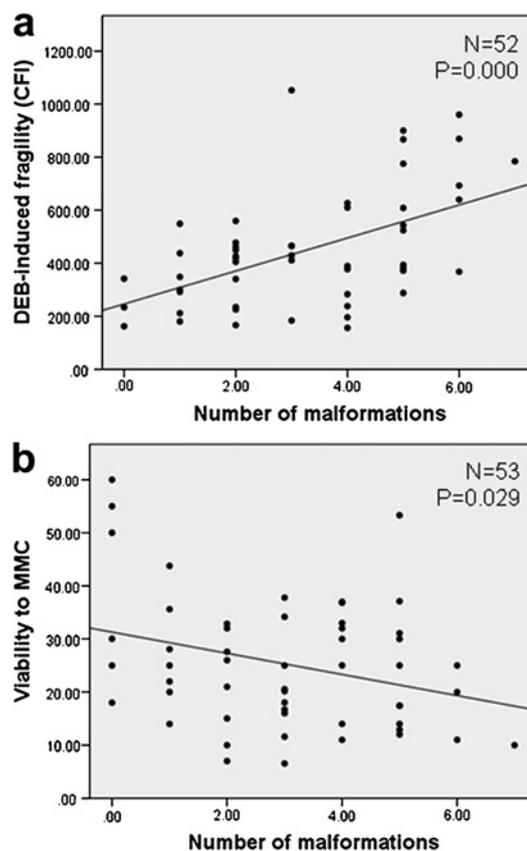


Figure 6 Correlations between number of congenital malformations and cell sensitivity to diepoxybutane (DEB) (Chromosome Fragility Index (CFI)) (A) and cell viability to mitomycin C (MMC) (B).

spontaneous chromosome fragility has been observed. This result indicates that both types of DNA damage are modulated by the same factors, and therefore, that spontaneous chromosome fragility is also a consequence of cellular inability to repair stalled replication forks probably induced by replication errors or endogenously produced ICL agents. How ICLs are generated endogenously is not yet clear, although products of lipid peroxidation seem to be able to cause this type of DNA damage.³⁰

Whether the clinical phenotype is directly caused by the deficiency in the repair of stalled replication forks is also an important question yet to be solved. ICL toxicity can explain cell proliferation deficiencies and why FA cells are prone to apoptosis. However, oxidative damage, telomeric dysfunctions, inflammatory response or replication stress can also explain part of the clinical phenotype.³¹ Interestingly, a good correlation between cell sensitivity to DEB or MMC and the number of congenital malformations was detected in this study. This result supports the hypothesis that cell death during embryonic development as a consequence of the cell inability to repair stalled replication forks, may be responsible for congenital malformations of patients with FA. DNA repair genes seem to have an important role during embryonic development, as congenital malformations are frequently seen in chromosome instability disorders, including Bloom, Nijmegen breakage syndrome or Seckel syndrome.³² The fact that this correlation was also significant when the analysis was restricted to FA-A patients was indeed expected as the vast majority of patients included in this study belong to this complementation group. However, it cannot be extrapolated to other genetic subtypes as there is increasing evidence that core complex and downstream FA proteins do not always function in a single unit or pathway.^{33–35} On the other hand, no relationship between cell sensitivity to ICLs and the age of onset of haematological disease has been detected. Haematopoietic cell progenitors are highly sensitive to pro-apoptotic cytokines that are expressed in bone marrow under stress conditions. Therefore, our data support the notion that development of BMF in patients with FA could be determined by factors other than DNA repair deficiency alone. Consistently, we have patients with FA with late onset of the blood disease but high sensitivity to ICLs and vice versa. Thus, this study suggests a model in which genome instability is necessary, but not sufficient, to induce early BMF in patients with FA. Uncovering the factors that modulate the age of onset and evolution of the haematological disease is a promising line of research aimed at ameliorating the haematological complications of FA.

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Contributors MC performed research, analysed data, and wrote the paper; RP, EC, MJR, JAC, MT, TF performed research; AM, JS, LM, EC, CB, CDdH, TO, JSdT, IB, JE, AD, AR-V, PG, MT, AM, and AF analysed data and contributed vital analytical tools and materials; JAB analysed data and JS coordinated the study, performed research, designed the research, analysed data and wrote the paper.

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