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Chromosome fragility in Fanconi anemia patients: diagnostic implications and clinical impact

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Abstract

Fanconi anemia (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 DEB or MMC is the gold standard test for the diagnosis of FA. In this study we present data from 198 DEB-induced chromosome fragility tests in non-FA and in FA patients where information on genetic subtype, cell sensitivity to MMC and clinical data were available. This large series allowed us to quantify the variability and the level of overlap in ICL-sensitivity among FA patients and normal population. In this article we propose a new chromosome fragility index that provides a cut-off diagnostic level to unambiguously distinguish FA patients, including mosaics, from non-FA individuals. Spontaneous chromosome fragility and its correlation with DEB-induced fragility was also analyzed, indicating that, while both variables are correlated, 54% of FA patients do not have spontaneous fragility. Our data reveal a correlation between malformations and sensitivity to ICLs. This correlation is also statistically significant when the analysis is restricted to the patients from the FA-A complementation group and suggests that genome instability during embryo development may be related to malformations in FA. Finally, chromosome fragility does not correlate with the age of onset of hematological disease, indicating that DEB-induced chromosome breaks in T-cells has no prognostic value in FA.

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Introduction

Fanconi anemia (FA) is a rare genetic disease characterized by bone marrow failure, congenital malformations, endocrine dysfunctions and cancer predisposition. It was first described in 1927 by the Swiss pediatrician Guido Fanconi[1] and its estimated incidence is about 1-5 cases in one million births in the overall population,[2] or up to less than 1 in 20.000 in some consanguineous ethnic groups.[3, 4, 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, 7, 8] In the USA, with 681 FA patients subtyped, FA-A is the most frequent complementation group representing 60.5% of the patients; FA-C and FA-G are also frequent, 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 4 out of 5 FA patients belonging to this complementation group.[11]

At 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 and co-workers described a high frequency of spontaneously-formed chromosome breaks in cells of FA patients.[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, 19, 20] Somatic mosaicism is produced when one of the pathogenic mutations is reverted in a hematopoietic precursor cell. Owing to an increased proliferative advantage, reverted cells are able to clonally expand and improve patient's blood counts. Depending on the stage of differentiation of the cell in which the gene correction occurred, reversion may affect all hematopoietic cell lineages, leading to a "natural gene therapy".[20, 21, 22] Alternatively, the reversion may affect only some hematopoietic lineages and therefore, it may not lead to an improvement of a patient's hematological 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 (% of cells with breaks or average number of breaks per cell) between non-FA and FA mosaics.[9]

The primary cause of death among FA patients is bone marrow failure (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 hematopoietic stem cell transplant (HST) from a compatible donor, which requires a previous conditioning of the patient. Regular conditioning regimens, consisting of chemotherapy and radiation, are highly toxic for FA patients, 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 HST.

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 FA patients 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 FA patients and normal population and, therefore, we propose a new chromosome fragility index that provides a clear cut-off diagnostic level to unambiguously distinguish FA patients (including mosaics) from non-FA individuals. Spontaneous chromosome fragility and its correlation with DEB-induced fragility is also analyzed 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 hematological 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 FA patients were obtained from their clinicians, including age of onset of hematological disease and number of congenital malformations. Number of congenital malformations was recorded as the well 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 consents were obtained according to the Declaration of Helsinki.

Chromosome fragility test

Chromosome fragility tests on peripheral blood lymphocytes were performed basically as described by Auerbach and co-workers,[17] with some minor modifications. Three blood cultures were prepared for each patient, including 0.5ml of blood in heparin and 4.5ml of culture consisting of 15% fetal bovine serum, 1% antibiotics, 1% L-Glutamine and 1% phytohemagglutinin in RPMI (all reagents from Gibco). Twenty four hours after culture set-up, two cultures were treated with DEB at a final concentration of 0.1µg/ml (Sigma, Cat.No.202533), and the remaining culture was left untreated for spontaneous chromosome fragility evaluation. 46h after DEB treatment, colcemid was added at a final concentration of 0.1µg/ml. Cultures were harvested 2h later when metaphase spreads were obtained following standard cytogenetic methods and finally stained with Giemsa. For chromosome fragility evaluation, 25-50 metaphases with 46 (1) centromeres were analyzed for each culture. The microscope analysis was performed with a Leitz Aristoplan microscope and, later with a Zeiss Imager M1 microscope coupled to a computer assisted metaphase finder (Metasystems, 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 a FA lymphoblastoid cell line to ensure that there was no significant variation between lots.

Analysis of cell survival to MMC

The survival of FA patient T cells to MMC 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) and anti-CD28 (CD28 Pharmingen, San Diego, CA) monoclonal antibodies. Five days later, the proliferating T cells were collected and exposed to increasing concentrations of MMC (0 to 1000nM) during additional five days in the presence of IL-2 (100u/ml, Proleukin, Chiron Corp, CA). Finally, the cells were re-suspended in PBS-BSA (0.05%) containing 0.5µg/ml propidium iodide (PI; Sigma) and incubated for 10 min at 4°C. Cell viability was determined by flow cytometry based on the PI exclusion test. In our previous study we observed that 33nM is the concentration of MMC that better discriminates between MMC resistant and sensitive T cells.[11] Therefore, survivals obtained after exposure to this concentration of MMC were routinely used to discriminate between T cells with a differential response to MMC.

Statistics

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

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 FA patients using exactly the same conditions. Of the 93 patients included in this analysis, 7 were of unknown complementation group and 86 were successfully subtyped. Of these, 70 FA patients 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 “% of aberrant cells”. As shown in Figure 1a and b, the number of “breaks/cell” is more than 10 times higher in the FA population when compared to the non-FA population, while “% of aberrant cells” increases 60 times in the FA group. However, the level of variability between FA patients is very high (see panels below Figures 1a and 1b) and, in some cases, chromosome fragility values in FA patients overlap with those observed in non-FA patients. Part of this high variability in the FA patients is due to the existence of a subgroup of FA patients who have lower values of “% of aberrant cells” and “breaks/cell” (Figure 1c and 1d). This subgroup corresponds to FA patients with a T-cell mosaicism. In this study, FA patients with less than 40% of aberrant cells are considered mosaic, while those with $\geq 60\%$ of cells are considered non-mosaic FA patients. FA patients 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 7 out of 8 lymphoblastoid cell lines generated from mosaic FA patients. All mosaic patients could be subtyped by retroviral

complementation, either in blood lymphocytes, when they were sensitive enough to MMC or in fibroblasts when they were resistant. In the latter case, 5 out of 5 established fibroblast cell lines showed the characteristic, MMC-induced G2/M phase cell cycle arrest and, therefore, were able to 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 2 or more breaks) (Figure 2b), chromosome fragility level of mosaic patients is equivalent to that observed in FA non-mosaics, even if a few cells with 2 or more breaks can be found in non-FA individuals (Figure 2b, lower panel). Therefore, neither “% aberrant cells” nor “breaks/cells” or “breaks/multiaberrant cell” indexes alone are enough to discriminate between non-FA and FA-mosaic patients. However, when combining “% of aberrant cells” and “breaks/cell” or “breaks/multiaberrant cell” in the same graph (Figures 2c and 2d, respectively), a better separation between patient subgroups is obtained. Thus, the data graphically presented in Figure 2d were transformed into a new index that we called “Chromosome Fragility Index” (CFI), resulting from multiplying the other two indexes. As shown in Figure 2e, CFI allowed establishing a cut-off value to clearly distinguish the non-FA from the FA population, including mosaics, without overlapping. The results of our study showed that non-FA patients have CFI values below 40, while all FA patients, including mosaics, have a CFI above 55.

The presence of tri- or tetra-radial figures is a characteristic feature of FA cells upon DEB treatment. In FA individuals, 1.373 multiradial figures were found in a total of 4.011 metaphases studied. Among all non-FA patients, 5.250 metaphases have been

studied and 3 cells with one multiradial figure were found in 3 different individuals (frequency of 0.057% or 1 in 1750 metaphases). Therefore, even though FA patients 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 has also been analyzed. Results obtained are described in Table 1. Overall higher spontaneous chromosome fragility is observed in FA patients (Figure 3a and 3b): 5.2 fold in “% 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 and 3b lower panels, spontaneous chromosome fragility interval values overlap in all 3 populations. Quantification of the proportion of FA patients (excluding mosaics) that present a level of spontaneous chromosome fragility in the range of non-FA population (Figure 3c), revealed that 54.4% of FA patients had spontaneous chromosome fragility within the normal range. In this graph, spontaneous chromosome fragility is measured by integrating “breaks/cell” and “%aberrant cells” in a “spontaneous chromosome fragility index” (SCFI), which results from multiplying “% of aberrant cells” and “breaks/cell”.

Finally, to further understand the mechanism that produces spontaneous chromosome fragility in FA patients, 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 FA patients (excluding mosaics) when using “% 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 were resistant to MMC while FA non-mosaics were highly sensitive, as expected. However, a wide range of MMC sensitivity is observed 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 to 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, 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 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 could 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 hematological disease. Mean age of onset of hematological disease for this population is 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 and 6b, cells from FA patients with a higher number of malformations statistically have more DEB-induced chromosome breaks ($P<.001$) 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 ($P<.001$). On the contrary, cell sensitivity to ICLs did not correlate with the age of onset of hematological 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 very laborious and requires specialized personnel. Among the different ICL inducers, DEB is commonly chosen, due to high compound stability and high specificity, as no other group of individuals with sensitivity to DEB comparable to FA patients has been described. To further improve our understanding of chromosome fragility in FA and find rational criteria to correctly and unambiguously diagnose FA patients, 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 FA patients and the non-FA population. The results on the chromosome fragility test

presented here have been obtained in an ethnically homogenous population of Spanish Caucasian individuals, and the cytogenetic 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 “% aberrant cells”) allow clear discrimination between 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 3 non-FA patients with one multiradial figure) can lead to a false positive diagnosis of mosaicism in a non-specialized laboratory. However, cells with breaks in FA mosaics are usually multiaberrant. This fact led us to develop a novel index (CFI) that integrates “% 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 to separate FA patients (including mosaics) from non-FA population. While it is possible that this cut-off level can vary between laboratories, we believe that the implementation of the CFI in diagnostic laboratories is highly recommended as it adds to the integrated analysis of the cell distribution of chromosome fragility, for a better and more reliable diagnosis of FA.

FA mosaic patients are usually discriminated from FA non-mosaics depending on % of aberrant cells. However, the mosaicism phenomenon has a progressive nature and, therefore, the differentiation between FA mosaic and FA non-mosaic (full FA) is not

always possible. Using restrictive criteria, when only FA patients with 40% or less of aberrant cells are considered as mosaics, 18% of Spanish FA patients are mosaic. With a less conservative upper limit of 50% of aberrant cells, the incidence of mosaicism would increase to more than 20%. Similar criteria for the detection of mosaicism have been proposed for other specialized laboratories[9] 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, there are mosaic patients with 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 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, hemoglobin 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 % of aberrant cells of 64%, 59% and 69%, respectively. This observation suggests a lack of mosaicism in the lymphoid lineage of the hematopoiesis, 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 FA patients (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 FA patients 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, 28, 29] In this study we show that spontaneous and DEB-induced fragility, as well as cellular sensitivity to MMC are all highly variable among FA patients, but a good correlation between sensitivity to ICL-inducing agents and 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. ICLs 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, good correlation between cell sensitivity to DEB or MMC and the number of congenital malformations has been 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 FA patients. DNA repair genes seem to play 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, 34, 35] On the other hand, no relationship between cell sensitivity to ICLs sensitivity and the age of onset of hematological disease has been detected. Hematopoietic cell progenitors are highly sensitive to pro-apoptotic cytokines that are expressed in bone marrow under stress conditions. Therefore, our data supports the notion that development of bone marrow failure in FA patients could be determined by factors other than DNA repair deficiency alone. Consistently, we have FA patients with late onset of the blood disease but high sensitivity to ICLs and vice versa. Thus, this study suggests a model where genome instability is necessary, but not sufficient, to induce early bone marrow failure in FA patients. Uncovering the factors that modulate the age of onset and evolution of the hematological disease is a promising line of research aimed to ameliorate the hematological complications of FA.

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Table 1. Spontaneous and DEB-induced chromosome fragility test: Summary of results for FA and non-FA groups. FA patients are subdivided in 3 categories: FA non-mosaic, FA possible mosaic and FA mosaic.

Group	% aberrant cells			% multiaberrant cells			Breaks / cell			Breaks / multiaberrant cell		
	n	Mean	Interval	n	Mean	Interval	n	Mean	Interval	n	Mean	Interval
<i>Spontaneous chromosome fragility</i>												
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
<i>DEB-induced chromosome fragility</i>												
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

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

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

Number of patients included (n) and statistical significance (P) are shown.

Legend to figures

Figure 1. DEB-induced chromosome fragility in FA and non-FA groups expressed by “% aberrant cells” (a) and “breaks/cell” (b). Upper panels indicate mean±standard deviation (SD) and lower panels indicate range. Distribution of FA patients for “% aberrant cells” (c) and “breaks/cell” (d). “M” is mosaic and “No-M” is non-mosaic.

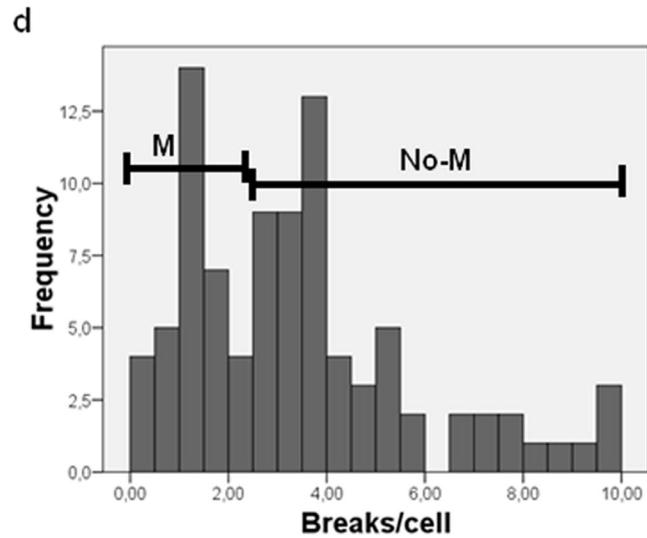
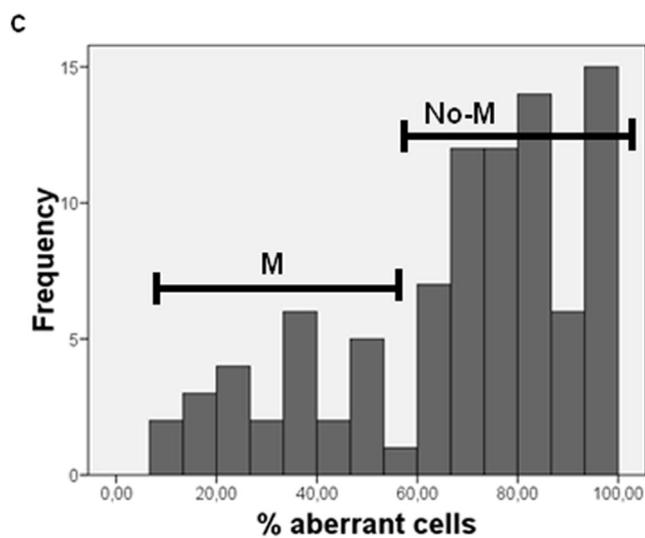
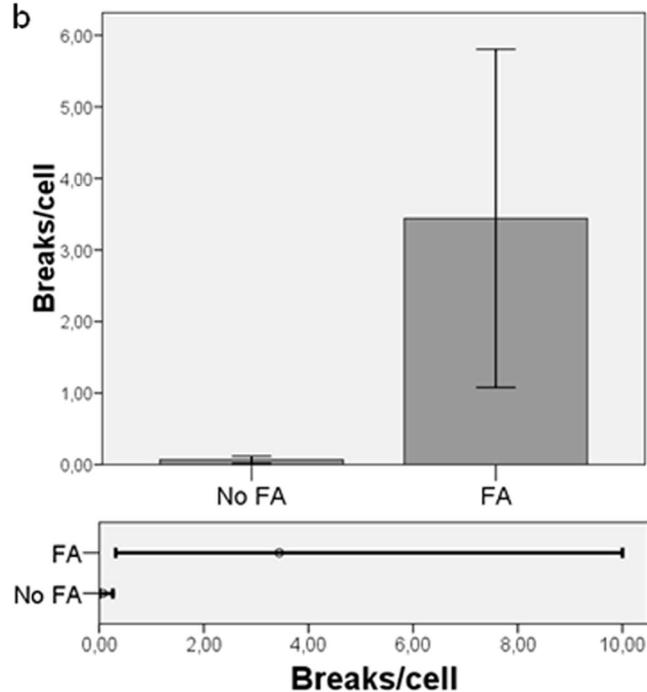
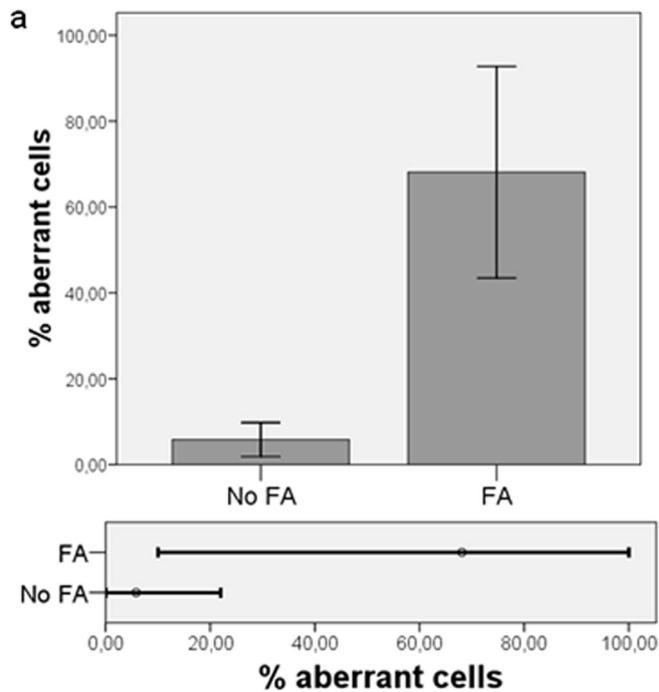
Figure 2. DEB-induced chromosome fragility in no-FA, FA mosaic and FA non-mosaic groups expressed by “breaks/cell” (a) and breaks/multiaberrant cell (b). Upper panels indicate mean±SD and lower panels indicate range. 2-D distribution of individuals analyzed regarding “% aberrant cells” and “breaks/cell” (c) or “breaks/multiaberrant cell” (d). Calculation of CFI for each group (e). Discontinuous line indicates the threshold (CFI=40).

Figure 3. Spontaneous chromosome fragility in no-FA, FA mosaic and FA non-mosaic groups expressed by “% aberrant cells” (a) and “breaks/cell” (b). Upper panels indicate mean±SD and lower panels indicate range. Statistical significance is shown. Accumulated percent of FA patients (mosaics excluded) regarding SCFI index (c). Reference line on X-axis indicates the upper level observed in non-FA population. Reference line on Y-axis indicates proportion of FA patients with SCFI below this limit.

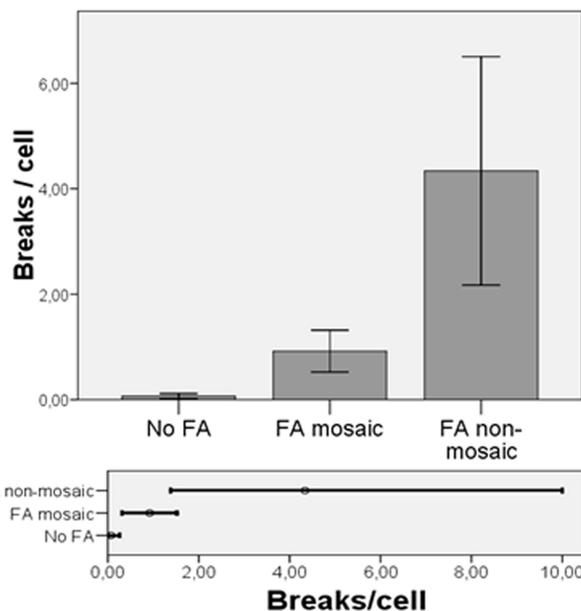
Figure 4. Correlation between spontaneous chromosome fragility and DEB-induced fragility expressed by “% aberrant cells” (a) and “breaks/cell” (b).

Figure 5. Cell viability to 33nM of MMC in no-FA, FA mosaic and FA non-mosaic groups (a). Upper panel indicate mean \pm SD and lower panel indicate range. Correlation between DEB-induced “% aberrant cells” and viability to MMC in mosaic patients; Shaded square indicates mosaic patients with less than 20% of aberrant cells that invariably show resistance to MMC (b). Correlation between DEB-induced fragility (CFI) and viability to MMC in FA non-mosaic patients (c).

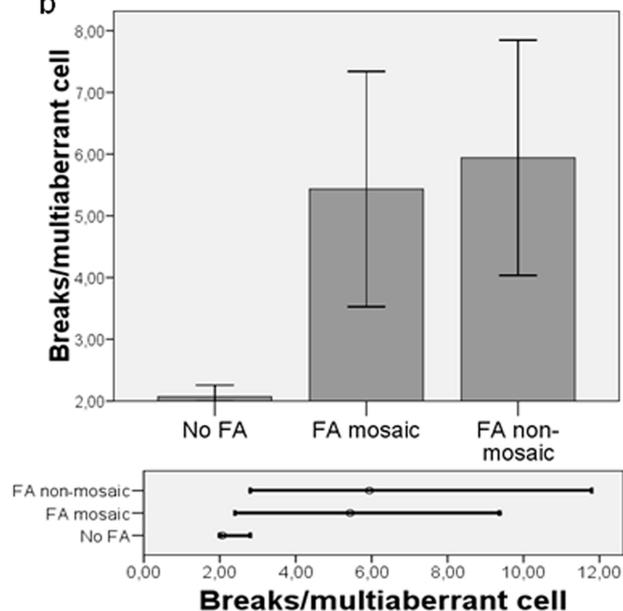
Figure 6. Correlations between number of congenital malformations and cell sensitivity to DEB (CFI) (a) and cell viability to MMC (b).



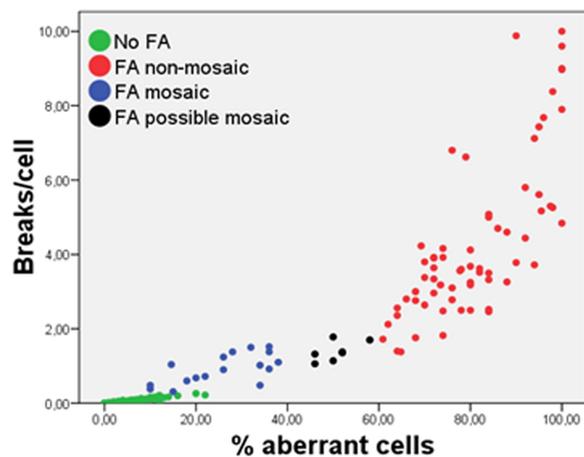
a



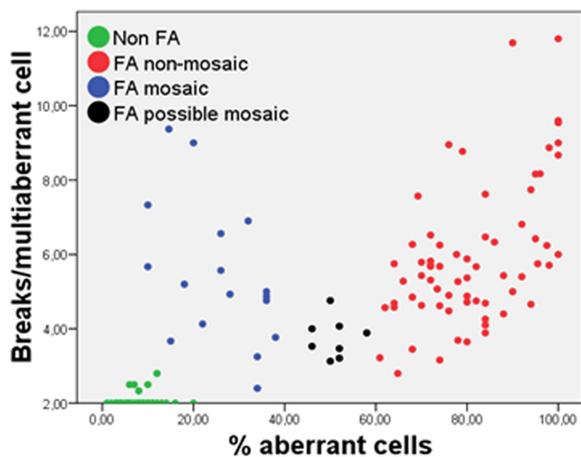
b



c

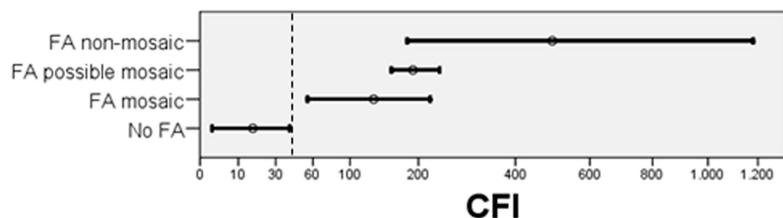


d



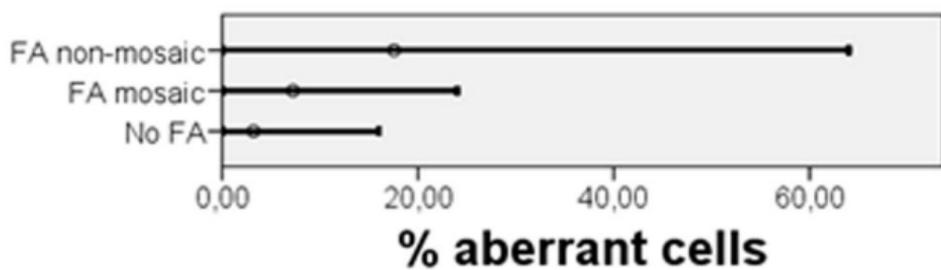
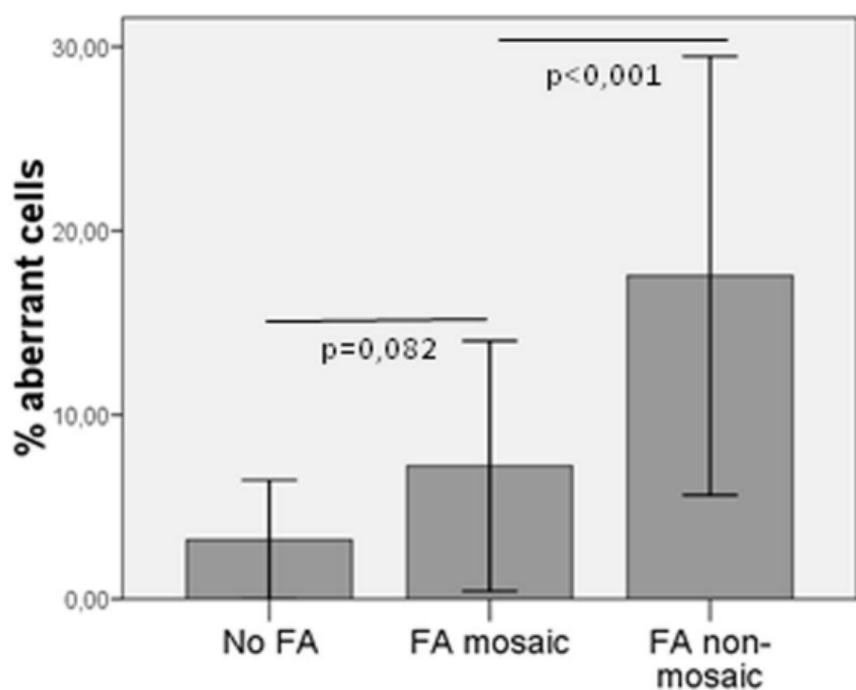
e

[CFI=(% aberrant cells)*(Breaks / multiaberrant cell)]

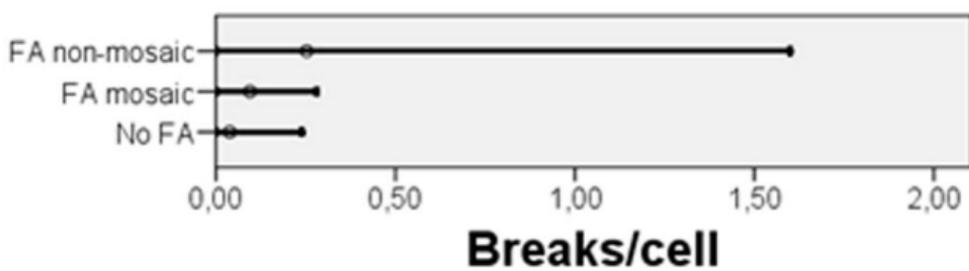
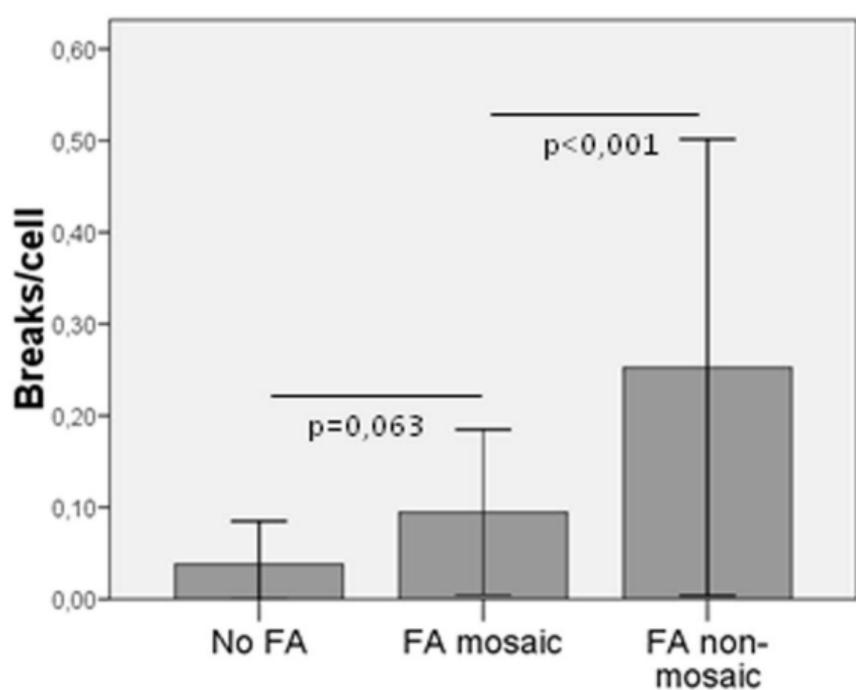


Interval
(181-1180)
(156-238)
(55-220)
(2-40)

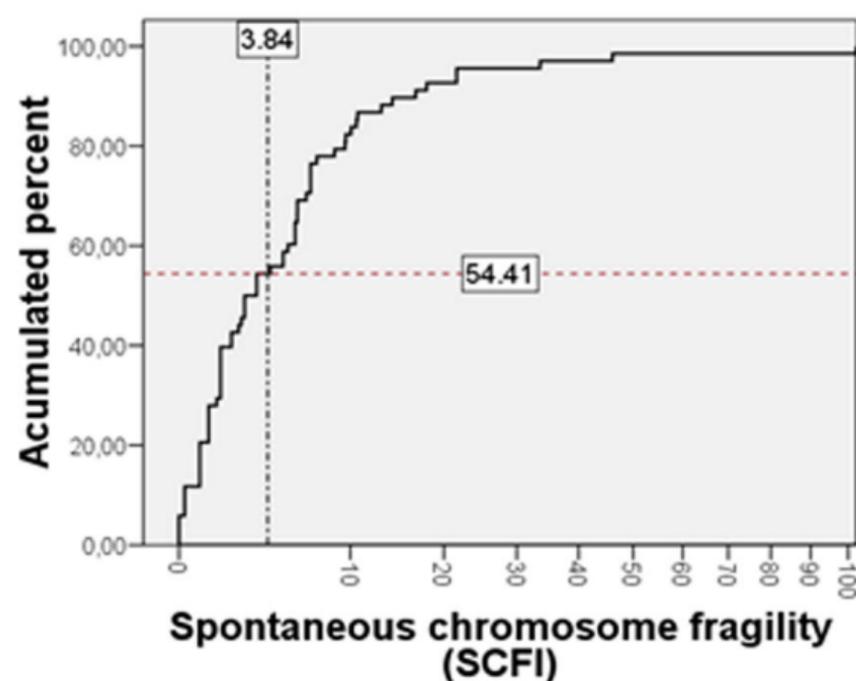
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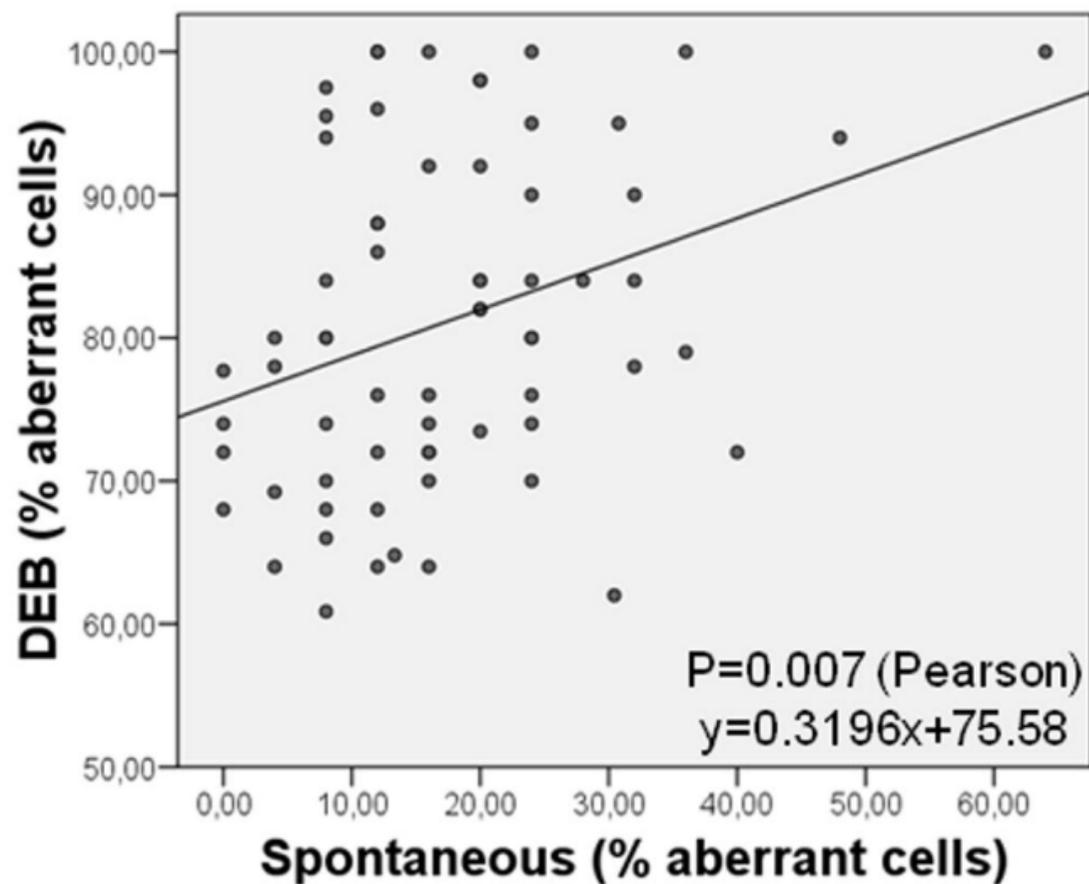
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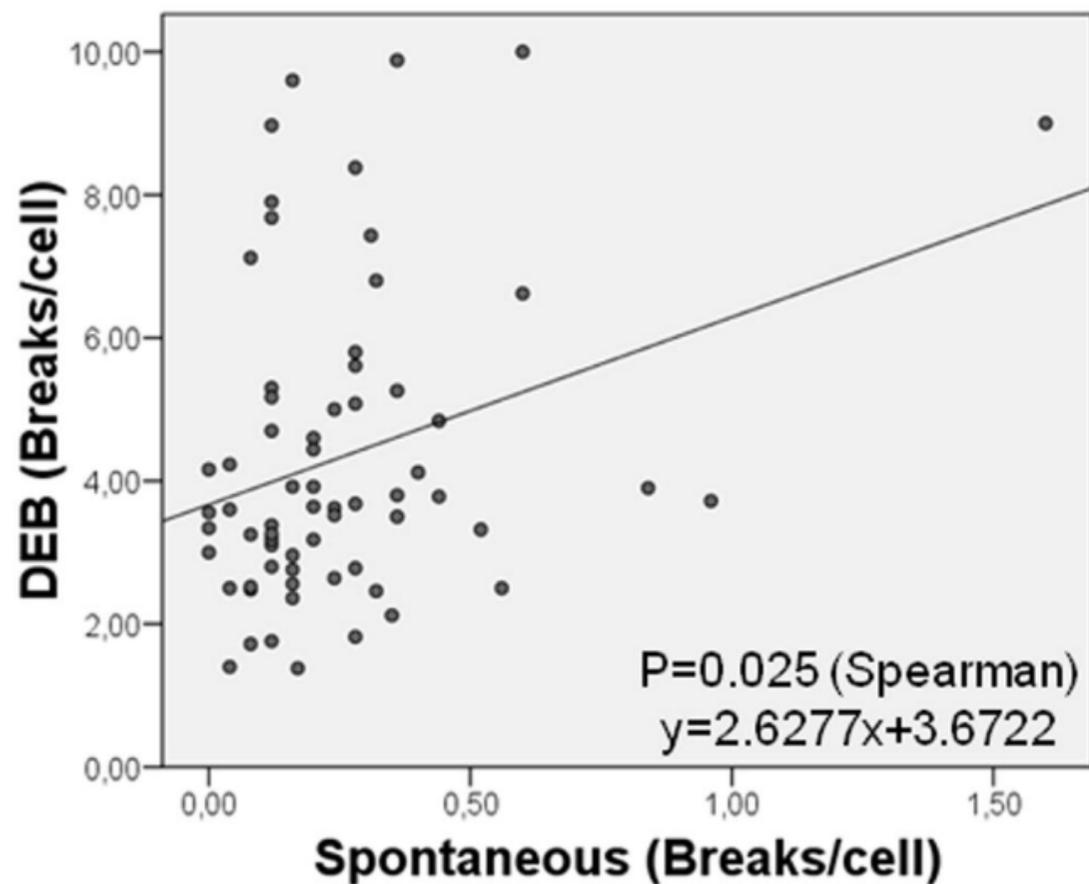
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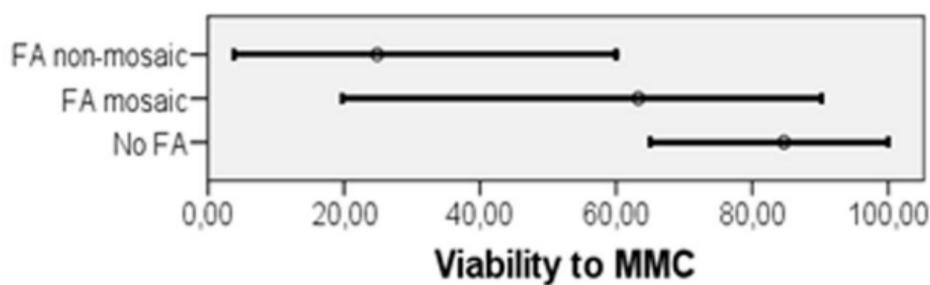
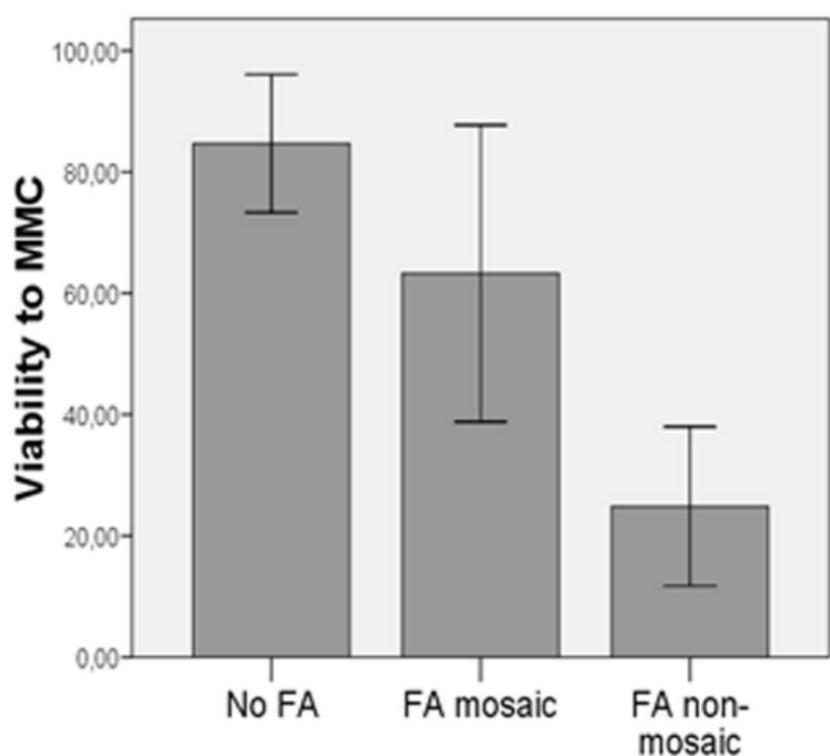
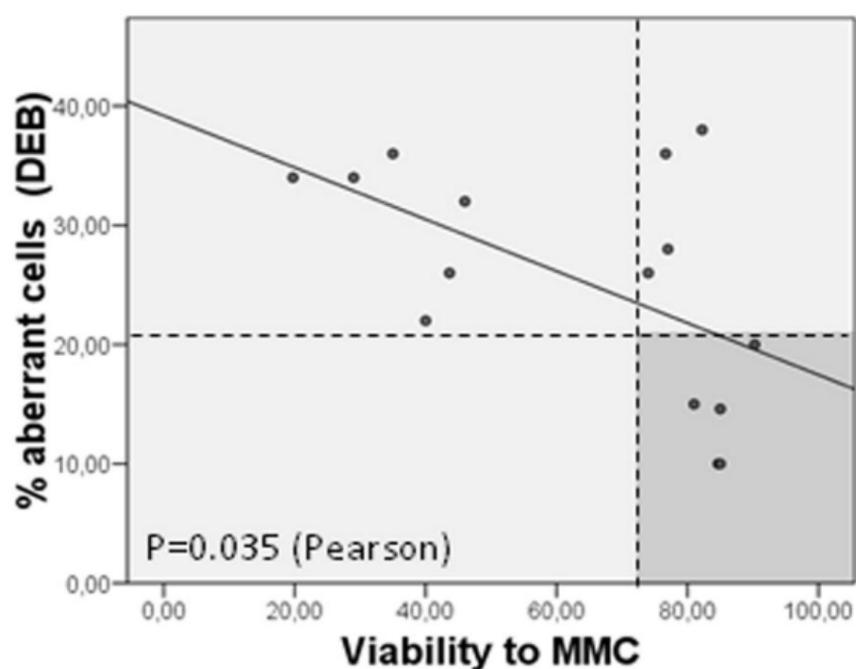
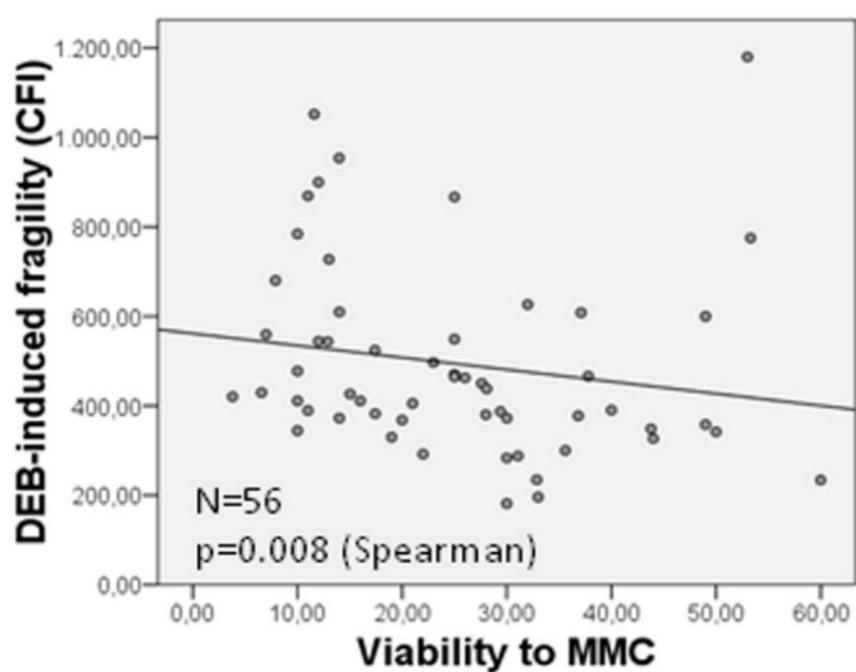


a

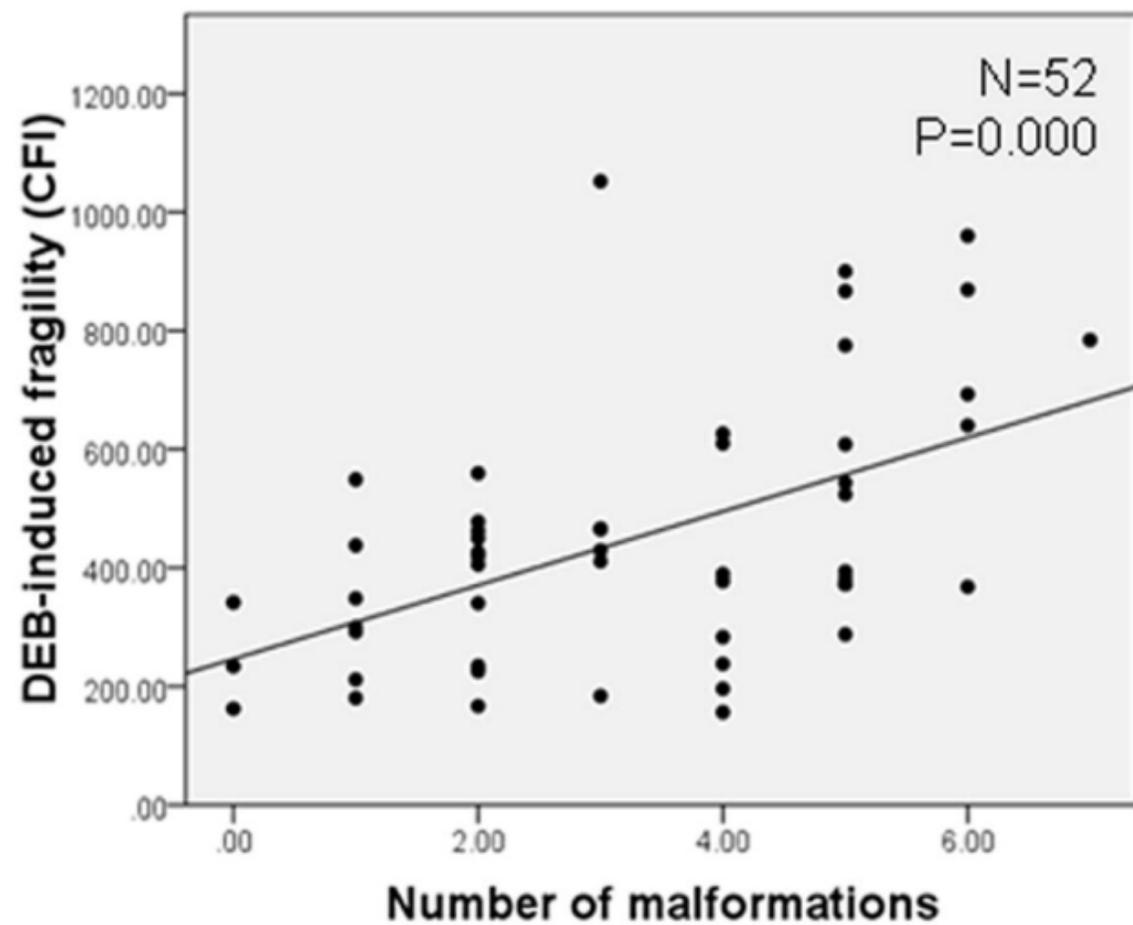


b



a**b****c**

a



b

