


# Use of the cell division assay to diagnose Fanconi anemia patients' hypersensitivity to mitomycin C

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## Funding information

ALF Research Grant; Barncancerfonden; Cancerfonden; Hjärt-Lungfonden; Stiftelsen Assar Gabrielssons Fond

## Abstract

The recently reported cell division assay (CDA) was optimized to measure the relative sensitivity of cells to cytotoxic drugs in vitro. Here, we investigated the in vitro hypersensitivity of lymphocytes from Fanconi anemia (FA) patients, to cytotoxic drugs using CDA. Peripheral blood mononuclear cells (PBMC) as well as cell lines derived from FA patients were treated with two DNA interstrand crosslinking (ICL) agents, mitomycin C and cyclophosphamide. Our data indicate that the CDA detects hypersensitivity of cells from FA patients to mitomycin C. Further, cell lines derived from FA-patients were also hypersensitive to mitomycin C as well as cyclophosphamide, when assayed by the CDA. This study suggests that the CDA is a useful alternative for the diagnosis of FA patients' hypersensitivity to ICL agents.

## KEYWORDS

cell division assay (CDA), DNA interstrand crosslinking (ICL) agents, Fanconi anemia (FA), flow cytometry, mitomycin C

## 1 | INTRODUCTION

We recently reported a cell division assay (CDA) to measure the relative sensitivity of cells to cytotoxic drugs in vitro (Mathew et al., 2016). The assay was developed as a possible tool in personalizing the dose of cancer therapies. Normal tissue toxicity in response to DNA-damaging cancer treatments is highly variable among patients (Safwat, Bentzen, Turesson, & Hendry, 2002; Tucker, Turesson, & Thames, 1992; Turesson, 1990). At least 80% of this variation has been attributed to heritable factors (Safwat et al., 2002). While the genetic factors resulting in this variation for most of the population is still unknown, patients with rare inherited disorders in DNA repair are known to be hypersensitive to specific types of DNA-damaging

therapies. Examples of DNA repair deficiency disorders include ataxia telangiectasia (AT) and Fanconi anemia (FA) (Ishida & Buchwald, 1982; Taylor et al., 1975). We previously showed that the CDA detects the hypersensitivity of AT patients to ionizing radiation, and an FA patient to mitomycin C (MMC) (Mathew et al., 2016). The ability to detect FA patient's hypersensitivity to DNA damaging treatment is of clinical importance for several reasons.

The diagnosis of FA is sometimes missed due to the genetic and phenotypic heterogeneity of the disease. Overall, an estimated 25%–30% of those affected have few or no abnormal phenotypes, and several of the clinical symptoms overlap with other diseases (Auerbach, 2009; Giampietro, Verlander, Davis, & Auerbach, 1997; Huck et al., 2006; Shimamura & Alter, 2010). To date, at least

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21 different genetic subtypes of FA are reported caused by a large number of pathogenic sequence variants (Flynn et al., 2014; Neveling, Endt, Hoehn, & Schindler, 2009). On the other hand, timely diagnosis of FA is critical for the clinical management of the patients. The current treatment for the hematological complications of FA is allogeneic hematopoietic stem cell transplantation (HSCT), which involves pre-treatment with ICL chemotherapy (Bonfim et al., 2016; MacMillan & Wagner, 2010), and needs to be modified for FA patients with respect to dose and choice of the treatment (Kelaidi et al., 2019; Torjemane et al., 2006). FA patients also have an increased risk of cancer development (Alter, 2003; Alter, 2014; Nalepa & Clapp, 2018), where ICL chemotherapy is often used in the treatment (Bonfim et al., 2016). Unfortunately, cases where the treatment with ICL chemotherapy precedes the FA diagnosis, leading to severe treatment-related side effects and morbidity, continue to be reported (Engel et al., 2019; Giampietro et al., 1993; Guan et al., 2018; Huck et al., 2006; Rochowski et al., 2012; Triemstra, Pham, Rhodes, Waggoner, & Onel, 2015).

Accordingly, there is a need for a quick and accurate assay to exclude FA before chemotherapy (Giampietro et al., 1993). Chemotherapy can be adjusted for FA patients, which has led to improved patient survival (Bonfim et al., 2016; Ebens, MacMillan, & Wagner, 2017; Goldsby, Perkins, Virshup, Brothman, & Bruggers, 1999; Gyger et al., 1989). FA results from a defect in the FA DNA repair pathway, which is required for the repair of DNA damage induced by ICLs (Deans & West, 2011; Walden & Deans, 2014). Thus, FA cells are characterized based on their profound sensitivity to ICL agents (Oostra, Nieuwint, Joenje, & de Winter, 2012). Today, the first step in the FA diagnosis is often based on measurement of chromosomal breakage in patient blood lymphocytes cultured with ICL agents (Auerbach, 2015; Oostra et al., 2012). The assay is carried out by a limited number of labs due to its technical difficulty, which involves visualization and scoring of chromosomes from cultured blood T cells treated with ICL. Notably, other methods of FA diagnosis using ICL sensitivity have been reported (Francies et al., 2018; Nalepa & Clapp, 2014; Seyschab et al., 1995), but are not used in clinical routine to our knowledge. A definitive molecular diagnosis of FA requires genetic testing. However, in some cases the functional assessment of ICL hypersensitivity of cells from FA patients is required to establish that the detected genetic aberrations are in fact pathogenic.

We recently reported the CDA as a method to measure the relative sensitivity of cells to cytotoxic drugs in vitro (Mathew et al., 2016). The assay uses incorporation of 5-ethynyl-2'-deoxyuridine (EdU) in the DNA in cultured cells to detect dividing cells (Rostovtsev, Green, Fokin, & Sharpless, 2002; Salic & Mitchison, 2008). The CDA was optimized to correlate with the clonogenic survival assay (Mathew et al., 2016), thereby serving as a surrogate for cell survival. The assay is suitable for clinical applications and can be carried out on fresh blood samples in 5 days (Mathew et al., 2016).

Here, we show that the CDA detects the in vitro sensitivity of cells and cell lines from FA patients to two ICL agents, MMC and cyclophosphamide.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and culture conditions

Epstein Barr virus-transformed lymphoblastoid cell lines (LCLs) from FA patients were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research (NJ, USA), and are listed in Supplementary Data. The cells were maintained in RPMI 1640 medium supplemented with 15% fetal calf serum, 100 U penicillin ml<sup>-1</sup> and 100 µg streptomycin ml<sup>-1</sup> (Sigma-Aldrich).

### 2.2 | Patient samples

Blood was taken in EDTA tubes from three patients with Fanconi anemia, and written informed consent was obtained. All blood samples were processed within 12 h. A short description of the patients is included in Supplementary Data.

Excess blood (EDTA tubes) from individuals with normal differential blood count was collected from the Sahlgrenska University Hospital central laboratory. Control samples were taken on the same day as the patient samples. Controls used for each experiment were assigned an arbitrary number and differ between different experiments. The study has been approved by the Regional Ethical Review Board in Gothenburg (Dnr: 938-16) and Swedish Ethical Review Authority (Dnr: 2019-01556).

### 2.3 | Drug treatment

A stock solution of 4-hydroperoxy cyclophosphamide (Santa Cruz) (50 µM), was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored in aliquots at -80°C. Mitomycin C (MMC) (12 mM) (Sigma-Aldrich) was prepared in DMSO and stored at 4°C for a maximum of 3 months. Drugs were diluted in culture medium and added to the cell suspension at the indicated concentrations.

### 2.4 | Cell division assay (CDA)

For blood samples, CDA was carried out as described previously (Mathew et al., 2016). A brief description of the method is included in Supplementary Data.

### 2.5 | Statistical analysis

Statistical analyses were carried out using the GraphPad prism 8 software (GraphPad Software). The p-values and significance cut-offs are outlined in Table S2.

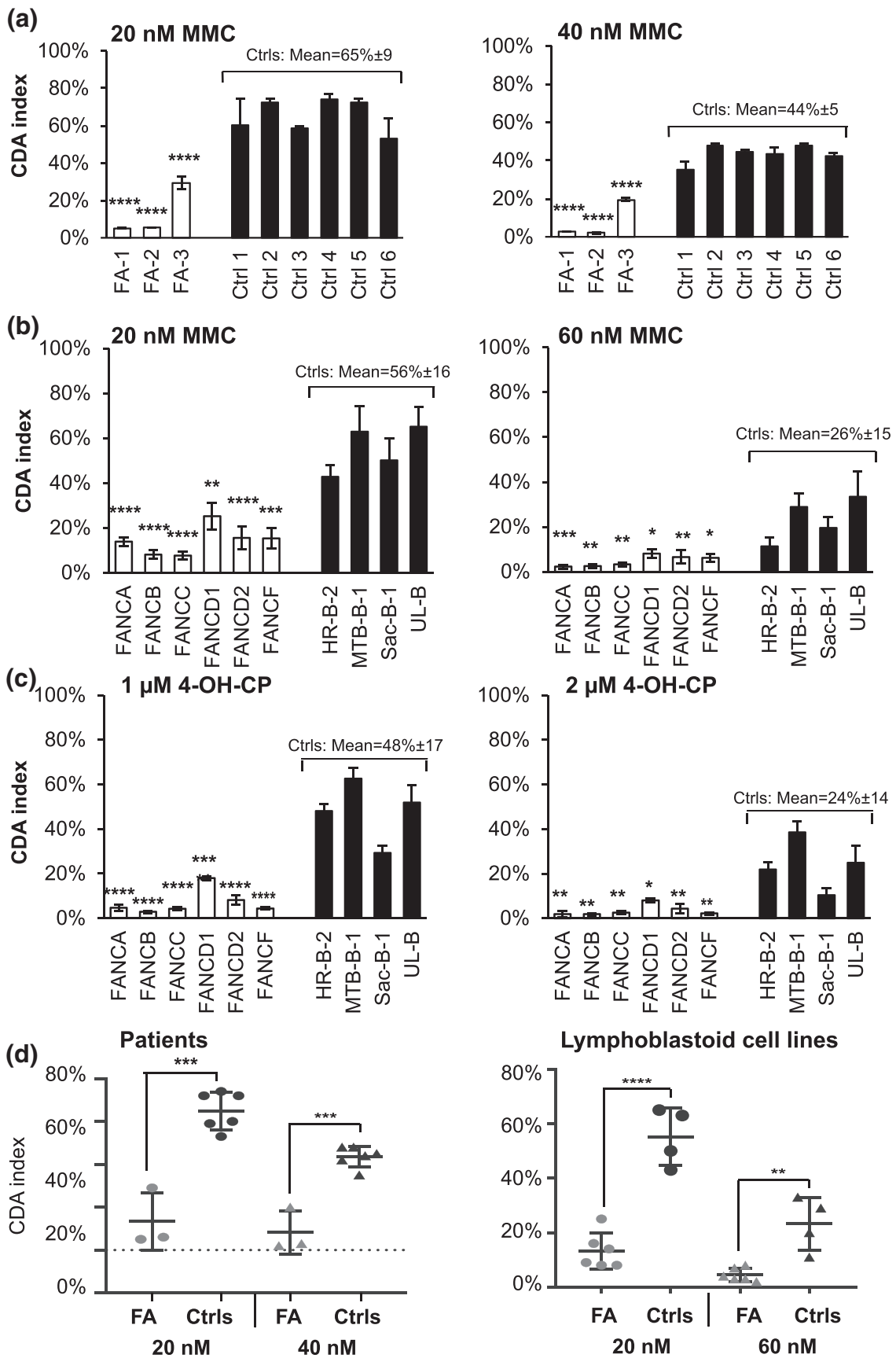


FIGURE 1 Legend on next page.

### 3 | RESULTS

#### 3.1 | Hypersensitivity to mitomycin C among patients with FA measured by the CDA

Peripheral blood mononuclear cells (PBMC) from three patients with aberrations in the FANCA gene were treated with mitomycin C (MMC) and assayed using the CDA. MMC concentrations were optimized to achieve a CDA index within the range of 70%–20% for normal individuals which allows testing in the linear range. An MMC dose response for two control individuals is shown in Figure S1. Patients denoted as FA-1 and FA-2 suffered from bone marrow failure while FA-3 (a sibling to FA-1) did not have anemia. The cells from FA-1 and FA-2 patients were 12- to 15-fold more sensitive to MMC treatment, while the cells from FA-3 were two-fold more sensitive when compared to the average sensitivity of the controls (Figure 1a). There was a dose dependent increase in CDA-measured sensitivity for all individuals, and the relative pattern of sensitivity for the different individuals was similar for both doses.

Next, lymphoblastoid cell lines (LCLs) derived from patients with mutations in different FA genes (complementation groups) were treated with MMC, and assayed using the CDA. MMC doses were optimized for LCLs as described above (data not shown). The FA-LCLs were 4- to 12-fold more sensitive relative to the average sensitivity for control LCLs (Figure 1b). FA patients have shown severe adverse side effects to cyclophosphamide, which is also an ICL agent. To determine the specificity of the assay, the relative sensitivity of the LCLs to 4-hydroxycyclophosphamide (4-OH-CP) (active metabolite of cyclophosphamide), was also investigated. The 4-OH-CP hypersensitivity pattern mirrored the MMC sensitivity of the cell lines observed above (Figure 1c).

Finally, the hypersensitivity measured for the patients as a group was compared to the control group and shown to be significant at both doses of MMC used here (Figure 1d). Similar results were observed for the FA-derived cells lines in comparison to control cell lines (Figure 1d). Thus, our data indicate that the CDA detects the hypersensitivity of the FA cells to ICL agents.

#### 3.2 | Inter and intra-individual variation in mitomycin C (MMC) sensitivity using the CDA

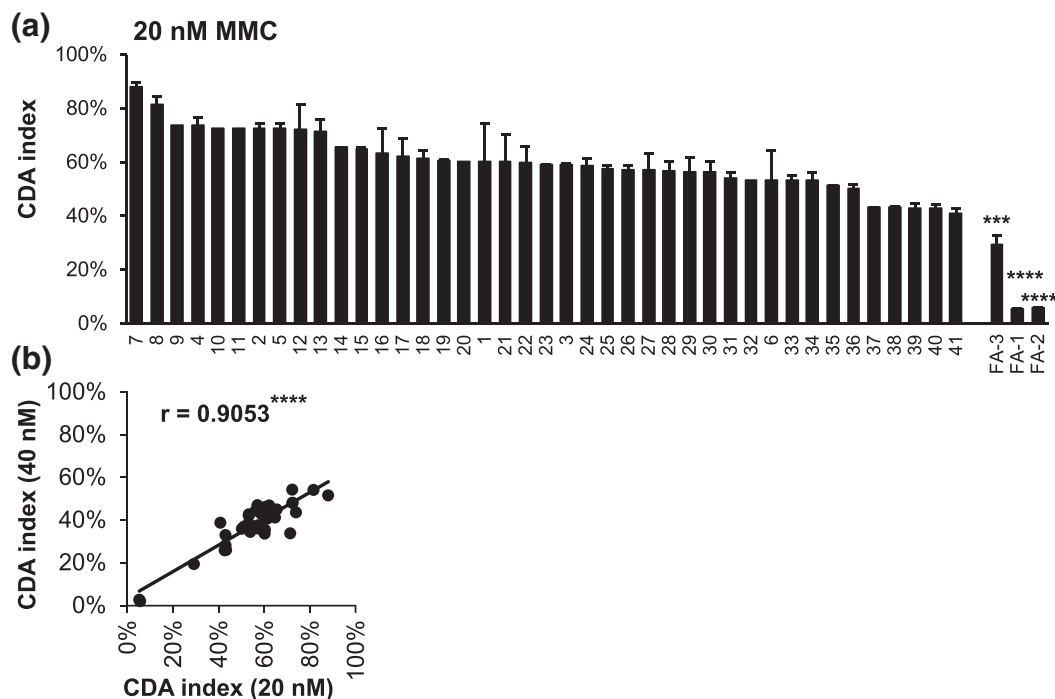
The variation in normal population sensitivity to MMC from was investigated using CDA (Figure 2a). The CDA index of 35 individuals,

as well as the six controls and FA patients from Figure 1 are presented as a bar graph. Notably, the CDA index is presented from the highest to the lowest in Figure 2a, and the additional controls included were given an arbitrary number from 7 to 41 in that order. Although the cells from FA patients were significantly hypersensitive to MMC relative to controls, there was a two-fold difference in the sensitivity to MMC between the control individuals (excluding FA) with highest and the lowest values. There was a significant linear correlation between the sensitivity at 20 and 40 nM MMC for these individuals (Figure 2b) indicating specificity of the assay, while there was no correlation between the sensitivity of the individuals and the rate of cell growth in the non-treated sample or the age of the individuals (Figure S2a,b). The intra-individual (day-to-day) variation of the CDA was measured by sampling 6 healthy individuals on three separate days (Table S1). The average day-to-day coefficient of variation of a single individual (intra-individual variation) was 4%–14% (with a mean of 10%) while the between-individual (inter-individual) CV measured for the 35 individuals was 17%–18%. It is likely that at least part of the intra-individual variation is due to the technical variation of the assay.

### 4 | DISCUSSION

We have previously described the CDA as a method for measuring the in vitro drug sensitivity of peripheral blood T cells in clinical routine (Mathew et al., 2016). Here, our data showed that the CDA detects the hypersensitivity of FA patients to MMC. This is in agreement with the finding for the FA patient reported in our previous study (Mathew et al., 2016). Interestingly, the patient genetically diagnosed with FA which did not suffer from bone marrow failure (FA-3) displayed a milder hypersensitivity to MMC in comparison to the other two FA patients, but was still significantly more sensitive than the controls when assayed using CDA. Thus, the sensitivity measured by the CDA appears to reflect the severity of the DNA-repair defect in FA patients. Since most FA patients undergo hematopoietic stem cell transplantation, it is not possible to carry out retrospective studies using their peripheral blood lymphocytes. We therefore made use of the EBV-immortalized lymphoblastoid cell lines (LCLs) established from FA patients to examine different FA complementation groups using the CDA. FA-derived LCLs have been shown to reflect the ICL hypersensitivity in vitro (Carreau, Alon, Bosnoyan-Collins, Joenje, & Buchwald, 1999) and are relatively genetically stable (Scheinfeldt et al., 2018), presenting a useful tool. In agreement with the MMC

**FIGURE 1** The cell division assay detects the sensitivity of FA cells to mitomycin C (MMC). (a) in vitro sensitivity of peripheral blood T cells from Fanconi anemia patients and healthy individuals using the CDA at 20 and 40 nM MMC. Error bars indicate the standard error of the mean of technical duplicates. The sensitivity of cells from each FA patient was compared with the sensitivity of control individuals using a two-tailed *t* test. (b) LCLs from FA patients and controls were treated with 20 and 60 nM concentrations of MMC and analyzed as above. Error bars indicate the standard error of the mean for at least three independent experiments. Each FA-derived LCL was compared with the average of control cell lines using a two-tailed *t* test. (c) The CDA index of the cell lines to 1 and 2  $\mu$ M 4-hydroperoxy cyclophosphamide (4-OH-CP) was carried out and presented as described in part B. (d) Scatter plots of the sensitivity to MMC in FA and controls as a group at 20 and 40 nM MMC (left panel) and MMC sensitivity in the FA-LCL and control LCLs at 20 and 60 nM (right panel). The average MMC sensitivity for FA cells and controls were compared using a two-tailed *t* test



**FIGURE 2** Inter-individual variation in mitomycin C (MMC) sensitivity using the CDA. (a) *in vitro* sensitivity of peripheral blood T cells from 41 healthy individuals and the three Fanconi anemia patients treated with 20 nM MMC using the CDA. Error bars indicate the standard error of the mean of the technical duplicates. The sensitivity of cells from each FA patient was compared with the sensitivity of these control individuals using a two-tailed *t* test. (b) Scatter plots showing the CDA index at 40 nM versus 20 nM MMC. *r* Represents Pearson's correlation coefficient (*p* value <0.0001, two-tailed)

hypersensitivity shown for cells from FA patients, the FA-LCLs were hypersensitive to MMC using the CDA regardless of their complementation group. Furthermore, treatment with the active metabolite of cyclophosphamide indicated an almost identical CDA hypersensitivity as with MMC in the FA-derived LCLs. Cyclophosphamide is a DNA-alkylating agent also resulting in ICLs and is used in the pre-treatment of HSCT and leukemia treatment. FA patients who were not diagnosed at the time of the cyclophosphamide treatment have been known to develop severe adverse side effects (Goldsby et al., 1999; Gyger et al., 1989). Overall, the low intra-individual variation of 10% in the control individuals, and the significant hypersensitivity of all the FA cells in response to ICL treatment, indicate that the CDA may be used to diagnose FA hypersensitivity. The error bars on the data obtained from cell lines sampled on several different days, further indicate the reproducibility of the assay. The presence of a biological difference in sensitivity to MMC among the healthy population suggests the presence of an inherent variation that may need to be taken into account before treating any patient with ICL therapy.

FA diagnosis prior to HSCT and cancer therapy, allows adjustment of the treatment and leads to improved long-term survival of FA patients (Bonfim et al., 2016; Ebens et al., 2017). The relative ease of CDA integration in diagnostic routine analysis, the short time-frame, intra-individual precision, and lower cost of the assay (Mathew et al., 2016) would allow screening of patients presenting with aplastic anemia, myelodysplastic syndromes, congenital abnormalities, childhood leukemia, or in need of HSCT. Thus, the CDA may be used as a

first step in the FA diagnosis. Patients with ICL hypersensitivity can then be followed up with genetic analysis for molecular diagnosis.

#### ACKNOWLEDGMENTS

We are grateful to the hematology staff and blood transfusion unit at the Laboratory of Clinical Chemistry at Sahlgrenska University Hospital for kindly providing us with control blood samples. We are grateful to the project student Anna McCormick for her assistance. We thank the staff at the "Komponentlaboratoriet" at Sahlgrenska University Hospital for their help with the radiation treatment. This study was funded by the Swedish Cancer Society (CF), the Swedish Heart and Lung Foundation, the Swedish Childhood Cancer Fund (BCF), Assar Gabrielsson's Cancer Research Foundation, and grants from the Swedish state under the agreement between the Swedish government and the country councils, the ALF-agreement.

#### CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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#### SUPPORTING INFORMATION

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

**How to cite this article:** Hammarsten O, Muslimovic A, Thunström S, Ek T, Johansson P. Use of the cell division assay to diagnose Fanconi anemia patients' hypersensitivity to mitomycin C. *Cytometry*. 2021;100:370–376. <https://doi.org/10.1002/cyto.b.21950>