# Diagnostic Accuracy of Brush Biopsy–Based Cytology for the Early Detection of Oral Cancer and Precursors in Fanconi Anemia

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**BACKGROUND:** Individuals with Fanconi anemia (FA) have a 500-fold to 700-fold elevated risk, much earlier onset, and limited therapeutic options for oral squamous cell carcinoma (SCC) compared with the general population. The early detection of SCC, or preferably its precursors, is mandatory to retain curative therapeutic options. Due to frequent synchronic and metachronic oral lesions, tissue biopsies, as usually recommended by guidelines, often are not feasible. In the current study, an alternative strategy for early detection using oral brush biopsy–based cytology was validated regarding its diagnostic accuracy. **METHODS:** Over a 12-year period, the oral cavities of a large cohort of 713 individuals with FA were inspected systematically and brush biopsy–based cytology of 1233 visible oral lesions was performed. In cases of inconclusive cytology, analysis of DNA ploidy was performed whenever possible. The results were correlated to a long-term clinicopathological follow-up reference standard. **RESULTS:** A total of 737 lesions were suitable for statistical analysis, including 86 lesions with at least high-grade oral epithelial dysplasia in 30 patients. For cytology, the sensitivity and specificity were 97.7% and 84.5%, respectively. Additional analysis of DNA ploidy increased the sensitivity and specificity to 100% and 92.2%, respectively. **CONCLUSIONS:** Careful inspection of the oral cavity of individuals with FA followed by brush biopsy–based cytology appears to identify visible oral, potentially malignant and malignant lesions that warrant treatment. Approximately 63% of SCC and precursor lesions are detected at a noninvasive or early stage. Negative cytology or a lack of DNA aneuploidy can exclude high-grade oral epithelial dysplasia or SCC with high accuracy and thus reduce the need for invasive diagnostic biopsies. ***Cancer Cytopathol* 2020;0:1-11**. © 2020 The Authors. *Cancer* Cytopathology published by Wiley Periodicals, Inc. on behalf of American Cancer Society. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

**KEY WORDS:** cytology; early detection of cancer; Fanconi anemia; image cytometry; oral cancer; sensitivity; squamous intraepithelial lesions.

## INTRODUCTION

Fanconi anemia (FA) is a rare disorder that is clinically characterized by the presence of specific malformations at birth; progressive bone marrow failure occurring mostly in early childhood; and an elevated risk of cancer,

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Valuable contributions to the oral cancer screening project were made by Kristiane Knops, Maike Hellmann, and Marietta Kazimirek from the Department of Cytopathology of the Institute of Pathology at Heinrich Heine University (Düsseldorf, Germany); Cornelia Sowa-Dietrich, Mona Markgraf, Christine Krieg, and Astrid Weiner-Schwarze from the German Fanconi Anemia Support Group; Kirstine la Cour Rasmussen from the Foreningen *Fanconi* Anæmi Danmark; and Suzanne Planck from the Fanconi Anemia Research Fund. We thank all 713 individuals affected by Fanconi anemia from all over the world who participated in this study, their treating physicians, and their local support groups who all contributed enormously to this project.

**Received:** August 19, 2019; **Revised:** December 14, 2019; **Accepted:** January 6, 2020

Published online Month 00, 2020 in Wiley Online Library (wileyonlinelibrary.com)

**DOI:** 10.1002/cncy.22249, wileyonlinelibrary.com

especially of the squamous epithelial type, during early adulthood.[1] On the cellular level, patients with FA demonstrate DNA fragility due to a defect in the DNA repair machinery[1] resulting from mutations in 22 genes that currently are known to cause the disease. Often, the diagnosis is made at the time of bone marrow failure. Nevertheless, some patients do not present with this typical clinical spectrum and thus diagnosis may be delayed or even missed.[2,3]

In the past, the main cause of death among patients with FA was impaired hematopoiesis.[4] Hematopoietic stem cell transplantation (HSCT) is the only curative therapeutic option for this feature of the disease. Due to better survival rates from successful HSCT, many patients with FA now are surviving into adulthood. At this stage, squamous cell carcinoma (SCC) is the main cause of death.[5,6] Unfortunately, patients with FA who are treated with HSCT demonstrate an elevated risk of developing SCC compared with those patients who do not undergo HSCT.[6] Moreover, the age of onset appears to be earlier. Head and neck SCC (HNSCC) is the most common type of SCC in patients with FA and approximately two-thirds of these cases are located within the oral cavity.[5] The risk of HNSCC increases with age, but compared with the general population, patients with FA develop these tumors at much earlier ages, with a median age at diagnosis in the early 30s. Many hypotheses, including human papillomavirus infection,[7] exposure to aldehydes,[8] and chronic infections,[9,10] have been investigated. However, to the best of our knowledge, the rationale for the 500-fold to 700-fold elevated risk of HNSCC in the FA population still has yet to be identified.

Due to underlying DNA repair defects, therapeutic options for SCC in patients with FA are limited. Standard chemoradiation protocols cannot be used among patients with FA due to severe therapy-related toxicities that lead to early death.[11,12] A cure is achievable but is limited to patients with SCCs at noninvasive or localized stages, which can be completely excised.[11,13] Moreover, patients with FA often present with frequent synchronic and metachronic tumors because they arise out of genetically altered fields.[14] When all these challenges are taken together, there is an urgent need for stringent surveillance in this special patient cohort. National guidelines have recommended frequent visits with experienced experts and tissue biopsies of every visible oral lesion that does not heal after 3 to 4 weeks.[15]

Nevertheless, data from the literature have demonstrated that the majority of HNSCCs in patients with FA are diagnosed at late stages, which leads to a poor prognosis.[11] This can be explained in part by the rarity of the disease and the atypical presentation of HNSCC in very young individuals without the main risk factors, such as exposure to known carcinogens (eg, tobacco and alcohol).[5] Moreover, many patients with FA, especially those who have undergone HSCT, present with multiple visible oral lesions that cannot all be biopsied. Thus, there is an urgent need for alternative screening strategies.

For the general population, there are many different minimally invasive strategies with which to characterize oral lesions.[16-18] Oral brush biopsy–based cytology for the noninvasive investigation of potentially malignant oral disorders in the general population has been proven to have high diagnostic accuracy.[17] In addition, DNA ploidy analysis may identify potentially malignant oral disorders with a high risk to transform to at least severe oral epithelial dysplasia (OED) (ie, to an indicator of malignant transformation).[19-25] Over a 12-year period (2006-2018), a large cohort of 713 patients with FA was screened using an approach that included noninvasive oral brush biopsy–based cytology. The current study reported on the diagnostic accuracy in this special cohort as a first-level diagnostic tool independently from the clinical presentation of a visible oral lesion.

## MATERIALS AND METHODS

### *Study Design and Participants*

The study was approved by the Western Institutional Review Board (study number 1139633) and by the ethics committee of the medical faculty of the Heinrich Heine University in Düsseldorf, Germany (study number 4168). All participants provided written informed consent.

Since 2006, individuals with FA were opportunistically recruited for the study through regional patient support group meetings, home visits, and local meetings in academic hospitals. Recruitment flyers were distributed to FA-related support groups and associations, research meetings and other events, hospitals, and academic institutions. Contact with the participants was mediated by regional FA patient support groups and their local treating physicians. Generally, all individuals with FA who agreed to participate were included and all patients with visible oral lesions underwent brushing. Due to a higher risk of bleeding during oral sampling, individuals with platelet counts <20,000/μL, bruising, or unusual bleeding in the

preceding 2 weeks prior to examination were excluded. All consented participants agreed to follow procedures dictated by the study protocol for follow-up. A standardized inspection of the oral cavity based on the World Health Organization oral cancer diagnosis protocol[26] with subsequent documentation of the location of visible lesions on an oral cavity map was performed at each visit. In addition, every lesion was documented digitally using a Karl Storz Endoscopic oral documentation system (Karl Storz IMAGE1 H3-Z Three-Chip-HD-Camera, HOPKINS Optic 0°, 10 mm, 20 cm; IMAGE 1 HUB HD Camera control unit; light source Xenon Nova 300; AIDA control NEO [Karl Storz SE, Tuttlingen, Germany]). Visible lesions were sampled using CerviBrushes (CellPath Ltd, Newton, United Kingdom) or Orcellex brushes (Rovers Medical Devices, Oss, The Netherlands) by spinning the brush at least 10 times on the lesion. Exfoliated cells were processed for liquid-based cytology (SafePrep [Medite, Burgdorf, Germany] or BD SurePath [BD Biosciences, Heidelberg, Germany]) or smeared on a microscopic slide and immediately fixed with alcohol spray (Merckofix; Merck, Darmstadt, Germany) for conventional cytology. All samples were analyzed as described below at the cytopathology department of the Heinrich Heine University in Düsseldorf, Germany. The cytological results were provided to the study investigators, who sent the data to the participants, including the oral cavity map indicating the location of any visible lesion and the digital picture of any visible lesion. In the event that suspicious cells were identified, participants were strongly encouraged to visit their local physician for further investigation. This early intervention approach was implemented to prevent the development of invasive SCC. Because some patients were examined repeatedly over the years, cytopathological results or clinical data from previous oral examinations were known to the investigators. Cytology results obtained from 2006 through 2018 were analyzed and correlated to a clinicopathological reference standard (as described below) to determine the diagnostic accuracy of oral brush biopsy–based cytology in individuals with FA.

### *Cytological Investigation*

Liquid-based oral brushings were vortexed, transferred to a 15 mL Falcon tube, and centrifuged (Rotina 56; Hettich, Tuttlingen, Germany) for 5 minutes at 500 × g at room temperature. The amount of the cell pellet was measured and the supernatant fluid was removed carefully with a pipette, leaving an excess of 1 mL (but no more than 2 mL total) per 5 µL of the pellet. Microscopic slides with 5 µL of the cell pellet each were prepared using a cytocentrifuge (Cellspin I; Tharmac, Wiesbaden, Germany) for 10 minutes at 1800 rpm at room temperature and subsequently air dried. Processed liquid-based specimens and conventional alcohol-fixed smears were stained according to the Papanicolaou method and interpreted by experienced/certified cytopathologists. Due to the lack of an international consensus guideline for reporting oral brush cytology,[27,28] all specimens were diagnosed according to the following diagnostic categories as previously reported: "negative" indicated normal squamous cells and reactive or inflammatory changes; "atypical" indicated atypical cells present (eg, superficial cell dyskaryosis); "suspicious" indicated dyskaryotic cells of the parabasal type or only a few malignant cells present; "positive" indicated malignant cells present; and "not sufficient" indicated few or poorly preserved squamous cells present or obscuring bacterial colonization.[29,30]

### *DNA Ploidy Analysis*

Specimens with atypical or suspicious cytology and, in a few rare cases, specimens with negative cytology but with a discrepant clinical impression, were analyzed for DNA ploidy using image cytometry as previously described.[29,31,32] Areas containing suspicious cells were marked on the coverslip of the specimen and photocopied to restore the labeling after uncovering and restaining according to Feulgen et al.[33] Measurements of nuclear DNA contents were performed using computer-based image analysis systems. During the years 2006 through 2010, a computer-based image analysis system consisting of a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany); a ×40 objective (numeric aperture, 0.75; Köhler illumination); a charge-coupled device black-and-white video camera (VariCam CCIR; PCO Computer Optics, Kehlheim, Germany); and the AutoCyte QUIC-DNA-Workstation software package (AutoCyte, Burlington, North Carolina) was used. Starting in 2011, a manual MotiCyte DNA workstation (Motic, Xiamen, China) consisting of a Motic BA400 microscope, a ×40 objective, a Moticam Pro 285A 12 bit color CCD camera, and MotiCyte-DNA image cytometry software was used. Both software packages provided shading and glare correction. All technical instruments and software used as well as the guidelines for diagnostic interpretation and quality assurance met the standard requirements of the

consensus reports of the European Society for Analytical Cellular Pathology (ESACP).[34-36]

For every measurement, at least 30 normal squamous epithelial cells, lymphocytes, or granulocytes were used for internal calibration (2c reference value). Approximately 300 (at least 100) nuclei of interest were selected in the previously labeled areas and the integrated optical density in c units (with c indicating nuclear DNA content) was measured and plotted in a DNA histogram. A DNA stemline was defined according to the ESACP recommendations as a population of cells with a similar DNA content accompanied by values approximately at its 2-fold DNA content. DNA aneuploidy denotes the DNA cytometric equivalent of chromosomal aneuploidy and indicates a malignant transformation of the measured population of cells.[21] DNA aneuploidy was indicated if the modal value of a DNA stemline in c units had a position outside normal peridiploid or peritetraploid ranges <1.80c or >2.20c and <3.60c or >4.40c, or if at least 1 cell with a DNA content of >9c did occur (single-cell aneuploidy). The latter criterion was applied only when accompanied by a population of cells with abnormal nuclear details such as enlargement, irregular shape, coarse chromatin structure, or hyperchromasia. This was necessary due to the euploid polyploidization that occurs in many different organs that exhibit inflammation, regeneration, and reactive changes,[37,38] such as lichen planus of the oral mucosa.[39] Euploid polyploidization is a benign condition with $2^n$ chromosomal sets (eg, tetraploidy) that has been recognized more intensely with the use of DNA cytometry techniques. In addition to the ESACP recommendations, a second diagnostic criterion for DNA cytometry was used if the interpretation of the measurement was inconclusive: a measurement was indicated as "suspected DNA aneuploidy" if an aneuploid DNA stemline consisted of only few cells (<60 cells) or if single-cell aneuploidy was accompanied by bland cytomorphological features in the Feulgen stain or if a stemline was very broad-based but with a modal value within the normal peritetraploid range. The latter was applied only if accompanied by the above mentioned abnormal nuclear features in the Feulgen stain.

This diagnostic criterion "suspected DNA aneuploidy" may distinguish oral SCCs that may have a near-tetraploid DNA content. DNA image cytometry was interpreted by experienced/certified cytopathologists.

### *Follow-Up Reference Standard*

Follow-up data of the oral lesions were collected either by direct communication with the participants and/or their treating physicians regarding the clinical course or again by oral inspection including brush cytology during long-term participation in the study. Follow-up information included the clinical status of the previously brushed lesion (eg, healing or persistence or progression of the lesion) as well as all available additional information, especially histopathological reports. The follow-up reference standard was defined in advance and data were collected that were blinded to the results of cytology and DNA image cytometry by an experienced pediatric hematologist-oncologist. The positive reference standard for an oral lesion was defined as a histological diagnosis of SCC or high-grade OED (including moderate and severe OED)[40] within 6 months of oral examination and cytology at the same region. In addition, a positive cytological diagnosis or the detection of DNA aneuploidy with a consistent clinical course (ie, SCC therapy, definite imaging, or palliative care) also was defined as a positive reference standard. The negative reference standard was defined as either a negative (benign) or low-grade OED histological diagnosis within 6 months or a negative clinical course within 2 years of oral examination and cytology at the same region.

### *Statistical Analysis*

Contingency table analysis of the categorical data (positive or negative) provided by both the reference standards and tests was used. Inconclusive test results (atypical or suspicious cytology) were classified as either positive or negative for the different statistical evaluations. The sensitivity, specificity, positive predictive value, and negative predictive value (NPV) with corresponding 95% CIs; area under the curve; and Fleiss kappa were calculated using SPSS statistical software (version 25; IBM Corporation, Armonk, New York).[41] The threshold level of significance was set to $P < .05$.

## RESULTS

### *Largest Cohort of Patients With FA*

From June 2006 to December 2018, a total of 713 individuals with FA were investigated for visible oral lesions. In total, 1233 evaluable brush biopsies were obtained from 279 individuals. One additional patient with FA intended to participate but was excluded due to

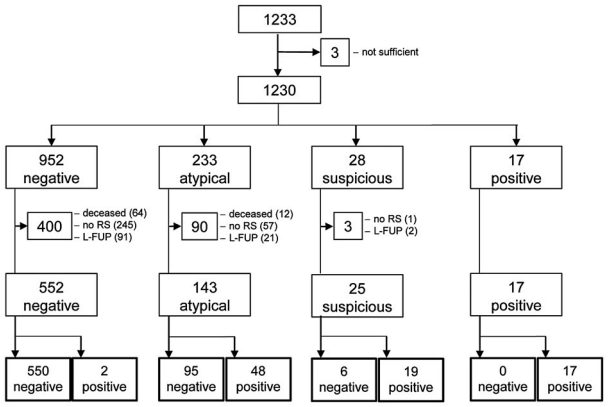


**Figure 1.** Flow diagram demonstrating patient recruitment and dropouts for cytology as well as the number of lesions undergoing cytological evaluation and the reference standard in the study period from June 2006 to December 2018. Boxes with bold lines indicate the results of the reference standard. L-FUP indicates lost to follow-up; not sufficient, material was not sufficient for cytological analysis; no RS, no reference standard was applicable.

low platelet counts. The results of the current study are reported per lesion if not otherwise indicated. Patient recruitment and dropouts, as well as the number of lesions undergoing cytological evaluation and the reference standard, are shown in Figure 1 as recommended by the Standards for Reporting of Diagnostic Accuracy statement.[42]

Adverse effects of oral brushings included sensation and mild bleeding that stopped rapidly and did not require further medical treatment. The specimens were classified as negative, atypical, suspicious, or positive based on cytological assessment. Three specimens were insufficient for diagnosis. DNA ploidy analysis was performed whenever indicated and possible as mentioned above. The above defined clinicopathological reference standard could be applied to 737 specimens. A total of 303 specimens did not meet the predefined requirements for follow-up criteria. No follow-up information was available for 190 specimens because patient contact was lost (114 samples) or patients had died (76 samples). The median time from brush biopsy to histological biopsy or oncological surgery was 1 month (range, 0-16 months). Detailed characteristics of the patient population, including demographic and clinical data, are summarized in Table 1.

Detection of oral cancer in early clinical stages such as Tis and T1 (according to the American Joint Committee on Cancer staging system[43]) and preferably at its precursor stage, histopathologically described as high-grade OED, is highly desired for individuals with FA. In the current study cohort, we identified 86 lesions among 30 individuals with FA with a positive reference standard. Of these individuals, 63% (19 of 30 individuals) were diagnosed in the desired early stages of disease with high-grade OED, including SCC classified as Tis (11 patients) or T1 (8 patients) (Table 2). All evaluable patients with ≥T2 oral SCC died of their oral tumor. Four patients with T1 oral SCC or high-grade OED died, but the cause of death was not found to be related to their oral disease.

### *Cytology of OEDs and SCCs in the FA Cohort*

For statistical evaluation, atypical and suspicious cytological results were classified as either "negative" or "positive" (Table 3). To make the application useful as a screening test for visible oral lesions, it was preferred that atypical and suspicious cytology be added to the "positive" statistical category to maximize sensitivity (variant 3; Table 3). For cytology, the sensitivity, specificity, positive predictive value, and NPV were 97.7%, 84.5%, 45.4%, and 99.6%, respectively. The kappa value of 0.548 indicated a moderate correlation of the cytological result and clinicopathological follow-up.

The cytomorphology of the oral squamous epithelium in the FA cohort does not differ from that of the general population. Normal cytological results include hyperkeratotic changes, ulcers and/or erosions, different degrees of mucosal inflammation, and candidiasis. Atypical cytological results include regenerating squamous epithelial cells that may demonstrate prominent nucleoli, hyperchromasia, coarse chromatin, and irregular nuclear contours with an inflammatory background and fibrin deposits (Fig. 2A), or superficial to intermediate cell dyskaryosis with a slight to moderately altered nuclear-to-cytoplasmic ratio and enlarged hyperchromatic nuclei (Fig. 2B). Suspicious cytology includes dyskaryosis of the parabasal cell type with a high nuclear-to-cytoplasmic ratio, irregular nuclear contours, and hyperchromatic nuclei or sometimes only very few malignant squamous cells in a specimen that are insufficient for a positive diagnosis. Specimens in the positive category demonstrate cytomorphology that is typical of an SCC with necrotic debris; scattered or small irregular groups of frankly malignant cells with cyanophilic cytoplasm; a high nuclear-to-cytoplasmic ratio; enlarged, hyperchromatic nuclei with nucleoli; and coarse chromatin (Fig. 2C Left). Keratinizing SCC may demonstrate bizarre configurated eosinophilic cytoplasm (Fig. 2C Right).

**TABLE 1.** Clinical and Demographic Characteristics of Study Sample

| Characteristics | All | Negative FUP | Positive FUP |
|---|---|---|---|
| Total no. | 737 | 651 | 86 |
| Median age at brush cytology (range), y | 27.3 (7.3-55.3) | 26.7 (7.3-53.6) | 30 (14.8-55.3) |
| Sex, F/M | 343/394 | 319/332 | 24/62 |
| Median follow-up (range), mo | 48 (0-149) | 48.7 (0-149) | 33.9 (0-126) |
| HSCT | 504 | 441 | 63 |
| Median age at HSCT (range), y | 8.8 (2.7-41.2) | 8.8 (2.7-41.2) | 8.8 (2.7-33.1) |
| Median age at brush cytology (range), y | 26.2 (7.3-51) | 25.8 (7.3-51) | 29.6 (14.8-39.9) |
| Sex, F/M | 207/297 | 194/247 | 13/50 |
| Median follow-up (range), mo | 56 (2-149) | 56.3 (4-149) | 49.3 (2-126) |
| no HSCT | 233 | 210 | 23 |
| Median age at brush cytology (range), y | 31.8 (8.9-55.3) | 31.8 (8.9-53.6) | 32.3 (24.8-55.3) |
| Sex, F/M | 136/97 | 125/85 | 11/12 |
| Median follow-up (range), mo | 35.9 (0-148) | 38 (0-148) | 13.6 (0-56) |
| Clinical diagnosis of lesion | | | |
| Leukoplakia | 405 | 403 | 2 |
| Erythroplakia | 81 | 76 | 5 |
| Erosion | 97 | 95 | 2 |
| Erythroplakia/erosion | 114 | 38 | 76 |
| Mixed lesion[a] | 40 | 39 | 1 |

Abbreviations: F, female; FUP, follow-up; HSCT, hematopoietic stem cell transplantation; M, male.
[a]Mixed lesion indicates erythroplakia, leukoplakia and erosion.

**TABLE 2.** Characteristics of Patients With Positive Clinicohistological Reference Standard[a]

| Histology/Cancer Stage[b] | No. of Patients | Median FUP, Months | Range, Months | Cause of Death |
|---|---|---|---|---|
| High-grade OED | 9 | 57 | 7-126 | Alive |
| Carcinoma, T1 | 6 | 38 | 6-78 | Alive |
| High-grade OED | 2 | 5, 34 | 33, 36 | BMF, other tumor[c] |
| Carcinoma, T1 | 2 | 35 | 16, 55 | BMF |
| Carcinoma, T2 | 3 | 20 | 17, 20, 49 | Oral SCC |
| Carcinoma, T3 | 2 | 13 | 12, 14 | Oral SCC |
| Carcinoma, T4 | 2 | 1 | 1, 1 | Oral SCC |
| Palliative[d] | 4 | 9 | 7, 9, 9, 56[e] | Oral SCC |

Abbreviations: BMF, bone marrow failure; FUP, follow-up; OED, oral epithelial dysplasia; SCC, squamous cell carcinoma.
[a]For patients with several oral lesions, only the lesion with the highest grade of OED or SCC stage was reported.
[b]Tis, T1, T2, T3, and T4 tumor categories were according to the American Joint Committee on Cancer staging system. High-grade OED included moderate and severe OED and SCC classified as Tis.
[c]Hypopharyngeal SCC.
[d]No biopsy was performed, clinically very advanced disease.
[e]Initial high-grade OED with disease progression.

**TABLE 3.** Diagnostic Accuracy of Oral Brush Biopsy–Based Cytology[a]

| | Variant 1: Atypical, Suspicious Indicates Negative | Variant 2: Atypical Indicates Negative; Suspicious Indicates Positive | Variant 3: Atypical, Suspicious Indicates Positive |
|---|---|---|---|
| Sensitivity[b] | 17/86 (19.8%) [1.4%-28.2%] | 36/86 (41.9%) [31.5%-52.3%] | 84/86 (97.7%) [94.5%-100%] |
| Specificity[b] | 651/651 (100%) [100%-100%] | 645/651 (99.1%) [98.4%-99.8%] | 550/651 (84.5%) [81.7%-87.3%] |
| PPV[b] | 17/17 (100%) [100%-100%] | 36/42 (85.7%) [75.1%-96.3%] | 84/185 (45.4%) [38.2%-52.6%] |
| NPV[b] | 651/720 (90.4%) [88.2%-92.6%] | 645/695 (92.8%) [90.9%-94.7%] | 550/552 (99.6%) [99.1%-100%] |
| AUC | 59.9% [52.8%-67.0%] | 70.5% [63.4%-77.6%] | 91.1% [88.5%-93.7%] |
| AUC *P* | .003 | <.001 | <.001 |
| Kappa coefficient | 0.303 | 0.526 | 0.548 |

Abbreviations: AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.
[a]Inconclusive (atypical and suspicious) cytology was classified as either statistically positive or negative, as indicated.
[b]The sensitivity, specificity, PPV, and NPV are presented as the number/total as a percentage with the 95% CI shown in brackets.

### *The Addition of DNA Image Cytometry Increases Diagnostic Accuracy*

DNA ploidy analysis with DNA image cytometry was performed whenever possible in cases with atypical or suspicious cytology. Of 168 specimens, 101 harbored sufficient cells for analysis (Fig. 3A). In addition, 11 cytologically negative specimens were analyzed due to a discrepancy between the negative cytological result and the clinical impression of the lesion (Fig. 3B). Of 17 cytologically positive specimens, 14 with positive follow-up

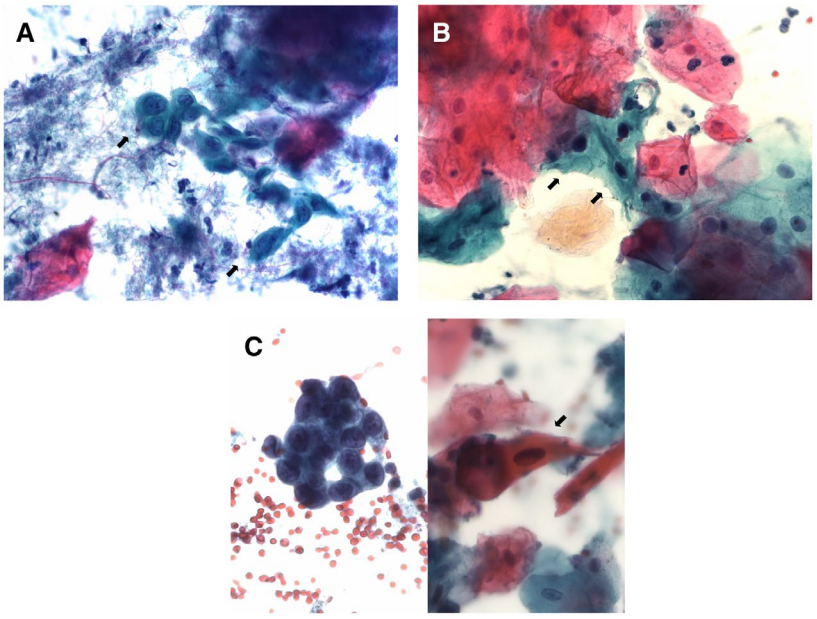


**Figure 2.** (A) Atypical cytology showing abnormal regenerating epithelium (arrows) with an enhanced nuclear-to-cytoplasmic ratio, irregular nuclear contours, and small nucleoli but finely dispersed chromatin. Bacteria is visible within the background of the specimen. (B) Atypical cytology showing superficial cell dyskaryosis (arrows) with an enhanced nuclear-to-cytoplasmic ratio and hyperchromatic, slightly enlarged nuclei. (C) Squamous cell carcinoma in a composite picture, demonstrating (*Right*, arrow) keratinized and (*Left*) nonkeratinized atypical cells with large, irregularly configured and hyperchromatic nuclei (Papanicolaou stain, original magnification ×40).

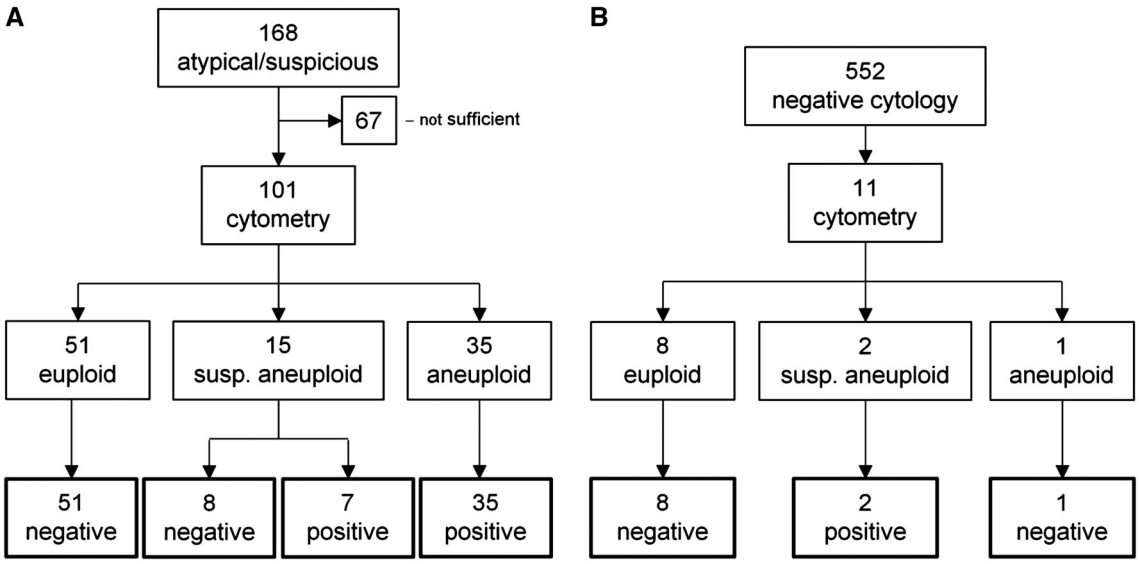


**Figure 3.** Flow diagram reporting the results of DNA ploidy analysis in (A) 168 atypical or suspicious and (B) 11 negative cytological samples. DNA ploidy analysis subsequent negative cytology was performed on clinical request. Boxes with bold lines indicate the results of the reference standard. Aneuploid indicates DNA aneuploidy; euploid, DNA diploidy or euploid polyploidy; not sufficient, material was not sufficient to be analyzed; susp. aneuploid, suspected DNA aneuploidy.

were eligible for DNA ploidy analysis, 13 of which were DNA aneuploid and 1 of which demonstrated an euploid polyploid histogram regarded as not DNA aneuploid. We measured cytologically positive specimens as positive controls because a cytological diagnosis is sufficient for further management and does not require analysis

of DNA ploidy for diagnostic confirmation. To enhance sensitivity, the category "suspected DNA aneuploidy" was included, as indicated earlier. The statistical results of all 126 DNA cytometric analyses compared with the follow-up, including the latter category as "positive," are shown in Table 4.

In the cytologically negative, atypical, or suspicious diagnostic categories, all euploid diploid or euploid polyploid DNA cytometric analyses were found to have negative follow-up results and 35 of 36 aneuploid DNA cytometric analyses had a positive follow-up result. The results for a combination of cytology and DNA ploidy for classifying the "suspected DNA aneuploidy" category as statistically either negative or positive also are shown in Table 4. The result of DNA ploidy analysis replaced a negative, atypical, or suspicious cytological result in the current evaluation. Incorporating DNA ploidy analysis into the cytopathological evaluation of oral brush biopsies increased the sensitivity and specificity to 100% and 92.2%, respectively.

## DISCUSSION

The detection and treatment of oral SCC in patients with early-stage disease has demonstrated favorable overall survival in the general population.[44] Unfortunately, only a minority of individuals with FA who develop oral SCC are diagnosed in the early stages of the disease.[11] Due to frequent synchronic and metachronic oral lesions in patients with FA,[14] invasive tissue biopsies, as usually recommended by national guidelines for the general population, in many cases are not feasible for individuals with FA. To our knowledge, the current study is the first to determine the use of brush biopsy–based cytology in investigating oral lesions among individuals with FA. This procedure is ideal because oral brushing is well tolerated by individuals with FA. The key benefit of the current study was its enormous sample size, especially within the context of a rare disease. An additional advantage of the current study was that oral brush biopsy with cytological investigation is a noninvasive, inexpensive, and simple procedure that can be replicated to a wider audience beyond the current study participants. The disadvantages of the current study were that the oral examinations and cytological investigations were performed by one workgroup and the screening approach was opportunistic and not population-based. Thus, it was not possible to estimate the NPV for an FA population-based cytological screening for OED or oral SCC.[45]

**TABLE 4.** Diagnostic Accuracy of DNA Ploidy Analysis Alone as Well as Oral Brush Biopsy–Based Cytology in Combination With DNA Ploidy Analysis (Calculating the Category "Suspected DNA Aneuploidy" as Either Statistically Negative or as Positive)

| | DNA Ploidy Alone; Suspected DNA Aneuploidy Indicates Negative N= 126 | Cytology Plus DNA Ploidy; Suspected DNA Aneuploidy Indicates Negative N= 737 | Cytology Plus DNA Ploidy; Suspected DNA Aneuploidy Indicates Positive N= 737 |
|---|---|---|---|
| Sensitivity[a] | 48/58 (82.8%) [73.1%-92.5%] | 77/86 (89.5%) [83.0%-96.0%] | 86/86 (100%) [100%-100%] |
| Specificity[a] | 67/68 (98.5%) [95.6%-100%] | 608/651 (93.4%) [91.5%-95.3%] | 600/651 (92.2%) [90.1%-94.3%] |
| PPV[a] | 48/49 (98.0%) [94.1%-100%] | 77/120 (64.2%) [55.6%-72.8%] | 86/137 (62.8%) [54.7%-70.9%] |
| NPV[a] | 67/77 (87.0%) [79.5%-94.5%] | 608/617 (98.5%) [97.5%-99.5%] | 600/600 (100%) [100%-100%] |
| AUC | 90.6% [84.6%-96.7%] | 91.5% [87.6%-95.4%] | 96.1% [94.8%-97.4%] |
| AUC *P* | <.001 | <.001 | <.001 |
| Kappa coefficient | 0.822 | 0.708 | 0.733 |

Abbreviations: AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.
[a]The sensitivity, specificity, PPV, and NPV are presented as the number/total as a percentage with the 95% CI shown in brackets.

Despite these limitations, examining the cytology and DNA ploidy of visible oral lesions in individuals with FA after a brush biopsy procedure demonstrated high specificity and sensitivity. Using oral brush biopsy–based cytology as a screening test, the NPV in patients with visible oral lesions was 99.6%. One of the false-negative cytological lesions was localized on the right side of the tongue and presented as hyperkeratotic and ulcerative. The second lesion was an erythroplakia in a patient with multiple lesions, which was found to be SCC. Early detection of oral SCC precursor lesions in individuals with FA is highly desirable, and therefore establishing atypical and suspicious cytology within the "positive" statistical category in the current study allowed for maximum sensitivity. By adding DNA ploidy analysis whenever possible for atypical or suspicious cytology or on clinical request for negative cytology, the NPV and sensitivity were enhanced to 100% because the 2 cytologically false-negative samples demonstrated suspected DNA aneuploidy. Moreover, the addition of DNA ploidy helped to reduce the need for invasive tissue biopsies in 101 of 168 cytologically atypical or suspicious samples and improved the specificity from 84.5% to 92.2%. In our opinion, this justifies the application of DNA image cytometry in addition to cytology because a more definitive result than "atypical" or "suspicious" cytology is provided for a clinical action. Nevertheless, the diagnostic category "suspected DNA aneuploidy" is ill-defined and future studies with better defined criteria are necessary (eg, introducing a cutoff value of 5% for the coefficient of variation of a "broad" peritetraploid stemline).

It is important to note that none of the 279 individuals with FA who presented with visible oral lesions and were investigated with brush-based cytology developed oral cancer in morphologically normal mucosa (median follow-up in the negative group: 48 months [range, 0-149 months]). Thus, the results from the current study demonstrated that unfocused brush biopsies on normal oral mucosa are not recommended. This also was noted by Maraki et al.[21]

Due to the very high NPV of negative cytology in the current study, the need for invasive tissue biopsies can be reduced to only cytologically positive or (for a maximum sensitivity at least suspected) DNA aneuploid lesions and to atypical or suspicious lesions that demonstrate too few morphologically abnormal cells for a DNA cytometric analysis. Moreover, if the DNA ploidy analysis demonstrated a euploid polyploid DNA distribution, invasive tissue biopsies could be spared (59 lesions). Based on the cohort for the current study, this means that approximately 81% of invasive tissue biopsies (600 of 737 biopsies) could be avoided without a diagnostic disadvantage to the patients. These results suggest that oral brush biopsies with cytological and on-indication DNA cytometric examination of every visible oral lesion should be required for oral cancer screening in individuals with FA. National guidelines define those lesions as those that are nonhealing after 3 to 4 weeks or that demonstrate a suspicious clinical appearance.[15] A watch-and-wait strategy for patients with cytologically negative and not DNA aneuploid lesions may be appropriate. If tumor cells or DNA aneuploidy are present, a total excision in favor of a biopsy of the respective lesion and histological examination should be strongly considered.

### *Conclusions*

Visible oral lesions in patients with FA very often are multiple and demonstrate a chronic course. Therefore, invasive histological biopsies are not optimal for repeat investigations. We believe the current study is the first to demonstrate that oral brush biopsy–based cytology with DNA ploidy analysis is a highly sensitive and specific, noninvasive alternative that can be used to detect oral SCC and precursor lesions in individuals with FA. Concomitant to careful visual inspection of the oral cavity, the brush biopsy procedure identifies visible oral and potentially malignant lesions requiring treatment. Approximately 63% of all patients with SCC and precursor lesions were detected at a noninvasive or early stage using this opportunistic screening approach. Negative cytology or a lack of DNA aneuploidy excluded patients with high-grade OED or SCC with high accuracy and thus reduced the need for invasive diagnostic biopsies in the follow-up period.

## FUNDING SUPPORT

The study "Reducing the burden of squamous cell carcinoma in fanconi anemia" was funded by the German Fanconi Anemia Support Group (Eschau, Germany) and by the Fanconi Anemia Research Fund (Eugene, Oregon, Funder DOI: 10.13039/100002086).

## CONFLICT OF INTEREST DISCLOSURES

Eunike Velleuer, Ralf Dietrich, Natalia Pomjanski, Bruno Eduardo Silva de Araujo, Stefan Biesterfeld, and Martin Schramm report

grants from German Fanconi Anemia Support Group. (Eschau, Germany) (since 2006) and grants from the Fanconi Anemia Research Fund (Eugene, Oregon) (since 2013) for work performed as part of the current study. Isabela Karoline de Santana Almeida Araujo has received grants from German Fanconi Anemia Support Group (Eschau, Germany) and Fanconi Anemia Foundation Germany for work performed as part of the current study. Alfred Böcking has received a grant from Motic Company (Xiamen, China) (until 2009) for the development of instruments for DNA image cytometry for work performed as part of the current study. Isis Sroka made no disclosures.

## AUTHOR CONTRIBUTIONS

**Eunike Velleuer:** Conceptualization, funding acquisition, data curation, investigation, methodology, formal analysis, project administration, writing–original draft, and writing–review and editing. **Ralf Dietrich:** Conceptualization, funding acquisition, data curation, investigation, methodology, project administration, and writing–review and editing. **Natalia Pomjanski:** Investigation, methodology, and writing–review and editing. **Isabela Karoline de Santana Almeida Araujo:** Software, formal analysis, and writing–review and editing. **Bruno Eduardo Silva de Araujo:** Data curation, resources, and writing–review and editing. **Isis Sroka:** Project administration and writing–review and editing. **Stefan Biesterfeld:** Investigation, methodology, and writing–review and editing. **Alfred Böcking:** Conceptualization, investigation, methodology, and writing–review and editing. **Martin Schramm:** Conceptualization, data curation, investigation, methodology, formal analysis, writing–original draft, and writing–review and editing.

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