

# Cystic Fibrosis F508del Patients Have Apically Localized CFTR in a Reduced Number of Airway Cells

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**SUMMARY:** Present state of knowledge, mostly based on heterologous expression studies, indicates that the cystic fibrosis transmembrane conductance regulator (CFTR) protein bearing the F508del mutation is misprocessed and mislocalized in the cytoplasm, unable to reach the cell surface. Recently, however, it was described that protein levels and localization are similar between F508del and wild-type CFTR in airway and intestinal tissues, but not in the sweat glands. In this study, we used immunocytochemistry with three different anti-CFTR antibodies to investigate endogenous CFTR expression and localization in nasal epithelial cells from F508del homozygous patients, F508del carriers, and non-CF individuals. On average, 300 cells were observed per individual. No significant differences were observed for cell type distributions among CF, carrier, and non-CF samples; epithelial cells made up approximately 80% to 95% of all cells present. CFTR was detected mostly in the apical region (AR) of the tall columnar epithelial (TCE) cells, ciliated or nonciliated. By confocal microscopy analysis, we show that the CFTR apical region-staining does not overlap with either anti-calnexin (endoplasmic reticulum), anti-p58 (Golgi), or anti-tubulin (cilia) stainings. The median from results with three antibodies indicate that the apical localization of CFTR happens in 22% of TCE cells from F508del homozygous patients with CF ( $n = 12$ ), in 42% of cells from F508del carriers ( $n = 20$ ), and in 56% of cells from healthy individuals ( $n = 12$ ). Statistical analysis indicates that differences are significant among all groups studied and for the three antibodies ( $p < 0.05$ ). These results confirm the presence of CFTR in the apical region of airway cells from F508del homozygous patients; however, they also reveal that the number of cells in which this occurs is significantly lower than in F508del carriers and much lower than in healthy individuals. These findings may have an impact on the design of novel pharmacological strategies aimed at circumventing the CF defect caused by the F508del mutation. (*Lab Invest* 2000, 80:857–868).

Cystic fibrosis (CF) is an autosomal recessive condition caused by the disruption of biosynthesis or the function of a membrane cAMP-activated chloride ( $\text{Cl}^-$ ) channel, the CF transmembrane conductance regulator (CFTR) (Kerem et al, 1989; Riordan et al, 1989; Rommens et al, 1989). The disease is characterized by progressive lung disease (the main cause of morbidity and mortality), pancreatic dysfunction, elevated sweat electrolytes, and male infertility (Collins, 1992; Welsh et al, 1995). The predominant mutation is the deletion of a trinucleotide resulting in the loss of phenylalanine at position 508 (F508del) (Collins, 1992). Several heterologous expression studies provided evidence that F508del-CFTR is misprocessed and mislocalized in the cytoplasm, unable to reach its appropriate location as a cAMP-regulated  $\text{Cl}^-$  channel in the cell membrane (Cheng et al, 1990;

Dalemans et al, 1991; Gregory et al, 1991; Lukacs et al, 1994). These findings were corroborated by a few immunocytochemical studies focusing on the sweat glands (Kartner et al, 1992), the bronchial tissues (Engelhardt et al, 1992), or primary cultures of CF airway epithelia (Denning et al, 1992b). Indeed, only a few reports have studied the localization of CFTR in freshly isolated human airway cells. Two of these studies, based on biochemical evidence, suggested that F508del-CFTR may be appropriately located in CF epithelia (Sarkadi et al, 1992; Zeitlin et al, 1992). Another recent study (Kälin et al, 1999), applying both immunohistochemistry and immunoblot analysis to several fresh tissues, reported that normal protein levels, processing, and localization were observed for F508del-CFTR in the airway and intestinal epithelia, but not in the sweat glands, of F508del homozygous patients.

Because of its possible therapeutic implications, it is important to determine the exact cellular localization of F508del-CFTR in nonrecombinant systems. In the present study, we analyzed cells from the respiratory epithelium because the airways are the main target of CF. Culture conditions of airway epithelial cells, their proliferation, and possibly also their immortalization can influence the differentiation state, which in turn affects CFTR expression levels, traffic, and processing

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(Jacquot et al, 1993). For these reasons, we analyzed here freshly obtained cells (Brézillon et al, 1995; Sood et al, 1992). To determine the intracellular localization of CFTR protein, we used three different and well-characterized anti-CFTR antibodies (Abs).

## Results

### ***Evaluation of Cellular Types Recovered by Nasal Brushing***

Nasal brushing samples from non-CF individuals ( $n = 5$ ), F508del-carriers ( $n = 5$ ), and patients with CF who were homozygous for F508del ( $n = 5$ ) were May-Grünwald-Giemsa (MGG)-stained (see "Materials and Methods" section) to evaluate the cellular types recovered. Nasal brushing samples contained three major types of epithelial cells: ciliated cells (CC), nonciliated cells (NC), including secretory, and basal cells (BC), as well as inflammatory cells (I) and a small group of nonidentified cells (NI), as shown in Figure 1A for a non-CF subject. Samples from the patients with CF and the carriers (not shown) had similar morphologic appearances.

The epithelial nature of cellular types was confirmed in all cases by immunostaining with anti-cytokeratin Ab (Fig. 1B). Epithelial cells accounted for approximately 80% to 95% of all cells (Fig. 1C), and ciliated cells were the predominant class (37%–42%). None of the differences observed for cell type distributions among the non-CF, carrier, and CF samples were significant.

### ***Specificity of CFTR Antibodies***

Several molecular and physiological tests performed by other authors demonstrated that CFPAC-1 cells express very low levels of CFTR and that CFPAC-PLJ-6-CFTR cells express CFTR in significant amounts, with some present at the membrane (Drumm et al, 1990). These two cell lines were used in this study (see "Materials and Methods" section) to confirm the specificity of the three anti-CFTR Abs by our immunocytochemistry protocol, before the analysis of nasal brushing samples (Demolombe et al, 1996). No CFTR was detected at the membrane of CFPAC cells by the 169 polyclonal Ab (Fig. 2A). However, this Ab could clearly detect CFTR, most of it at the membrane in CFPAC-PLJ-6-CFTR cells, demonstrating its specificity under our protocol. Similar results were obtained for both monoclonal Abs M13-1 and MATG 1061 (not shown). Controls in which the anti-CFTR Ab was omitted (not shown) were also negative.

### ***Localization of CFTR in Nasal Brushing Cells***

Shown in Figure 3 are nasal brushing samples from control subjects (panels A, C, and E) and F508del homozygous patients with CF (B, D, and F), immunostained for CFTR with three different anti-CFTR Abs (see "Materials and Methods" section): 169 (panels A and B), M13-1 (panels C and D), and MATG 1061 (panels E and F). Similar results were obtained for

samples from F508del carriers (not shown). As is evident from these microphotographs (*arrows* in Fig. 3), CFTR protein was mostly detected in the apical region (AR) of the tall columnar epithelial (TCE) cells (ciliated or not) in these samples. It is also evident that all three Abs, raised against three different regions of CFTR, recognize similar structures present in or near the apical membrane of these cells. However, this was not observed for all TCE cells (*arrowheads* in Fig. 3). A faint intracellular CFTR-labeling was also detected in basal cells. Surprisingly, TCE cells in CF samples also displayed CFTR-staining in their AR with all three Abs (Fig. 3, B, D, and F). The same occurred in samples from F508del carriers (not shown).

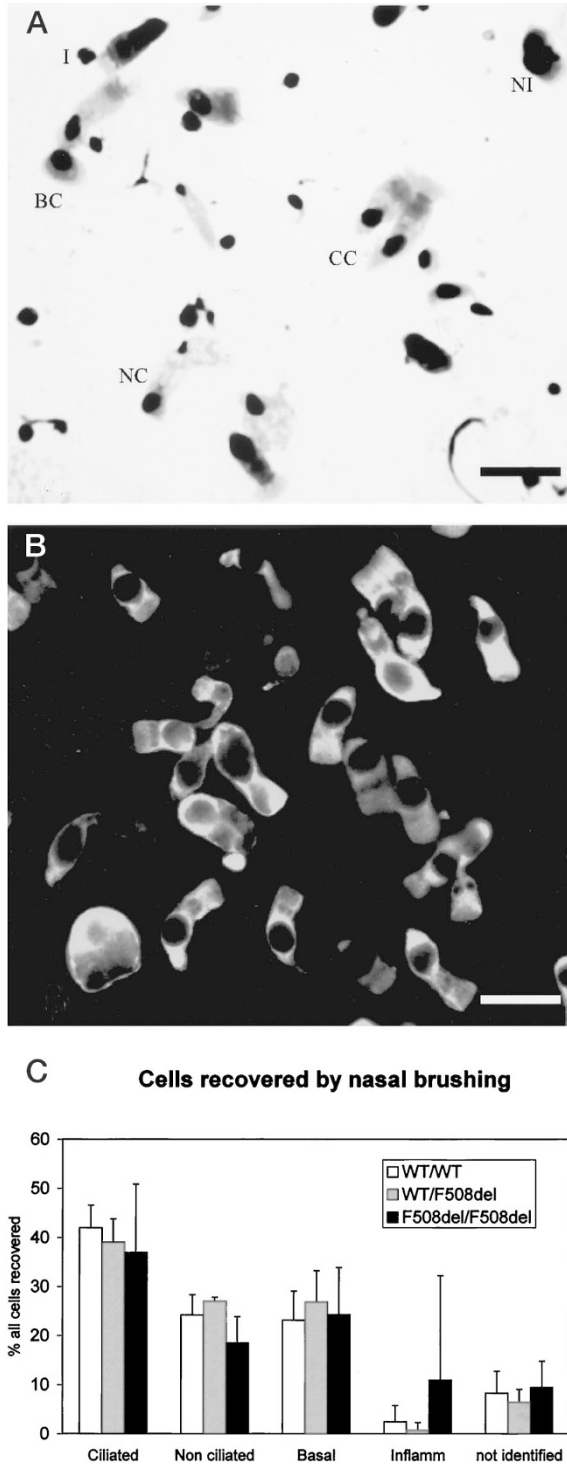
### ***Double Labeling of CFTR, Calnexin, p58, and Tubulin***

To confirm that the CFTR-staining did not result from unspecific adherence of Abs to the surface of TCE cells, particularly if ciliated, we carried out double immuno-labeling of nasal cells with polyclonal anti-CFTR 169 Ab (in green, *arrows* in Fig. 4, A-C, D-F, G-I, J-M, N-P, and Q-S), together with monoclonal anti-calnexin Ab (in red), staining mostly the ER (*arrowheads* in Fig. 4, B-C and E-F), monoclonal anti-p58 Ab (in red) staining the Golgi (*arrowheads* in Fig. 4, H-I and L-M), and monoclonal anti-tubulin Abs (in red) staining the cilia (*arrowheads* in Fig. 4, O-P and R-S).

It is evident from results in Figure 4 that neither the anti-calnexin Ab (panels B and E) nor the anti-p58 Ab (H and L) stains the surface of TCE cells as does the anti-CFTR 169 Ab (A and D), thus discarding unspecific CFTR-labeling in the AR. Clearly, some overlap occurs in labeling of CFTR and calnexin (see yellow in panels C and F), particularly for the F508del/F508del cells (in F). Some overlap also occurs with anti-CFTR and anti-p58 (yellow in panels I and M). Anti-CFTR-staining shows no overlap with the anti-tubulin Ab (no yellow in P and S), which distinctly stains the cilia of TCE cells (Fig. 4, O and R).

### ***Statistical Analysis of Tall Columnar Epithelial Cells with CFTR in the Apical Region***

The fact that CFTR was only detected in the AR of some TCE cells, in the three types of samples analyzed (non-CF subjects, carriers, and patients with CF), led us to count these cells. Therefore, for each nasal brushing sample, we assessed total TCE cells present and determined which of these had CFTR in the AR. Between 80 and 1,500 TCE cells were counted per individual and an average of 300 TCE cells per individual for all three groups. Altogether, we analyzed samples from non-CF subjects ( $n = 12$ ), F508del-carriers ( $n = 20$ ), and patients with CF homozygous for F508del ( $n = 12$ ). Results obtained with the three anti-CFTR Abs (Fig. 3) are shown in Table 1. Data in this table were subjected to various statistical tests, and the respective results are shown in Table 2 and in Figure 5. The differences among the averages of all three groups were statistically significant in all tests applied (see Table 2) and for all three Abs. The graphs



**Figure 1.**

Evaluation of cellular types recovered by nasal brushing. An example of an MGG-stained nasal brushing sample from a normal individual is shown in panel A. Three major types of epithelial cells are observed: ciliated (CC), nonciliated, including secretory (NC), and basal cells (BC). Inflammatory cells (I) and a small group of still not identified cells (NI) are also observed. The epithelial nature of cellular types was confirmed by immunostaining with anti-cytokeratin Ab (see "Materials and Methods" section) and is shown in panel B. Bar = 100  $\mu$ m. The morphologic appearance and cytokeratin-staining of samples from patients with CF and F508del carriers (not shown) are similar to that shown in panels A and B, respectively. The graph in C summarizes cell-type distribution in samples from non-CF subjects, carriers, and patients with CF.

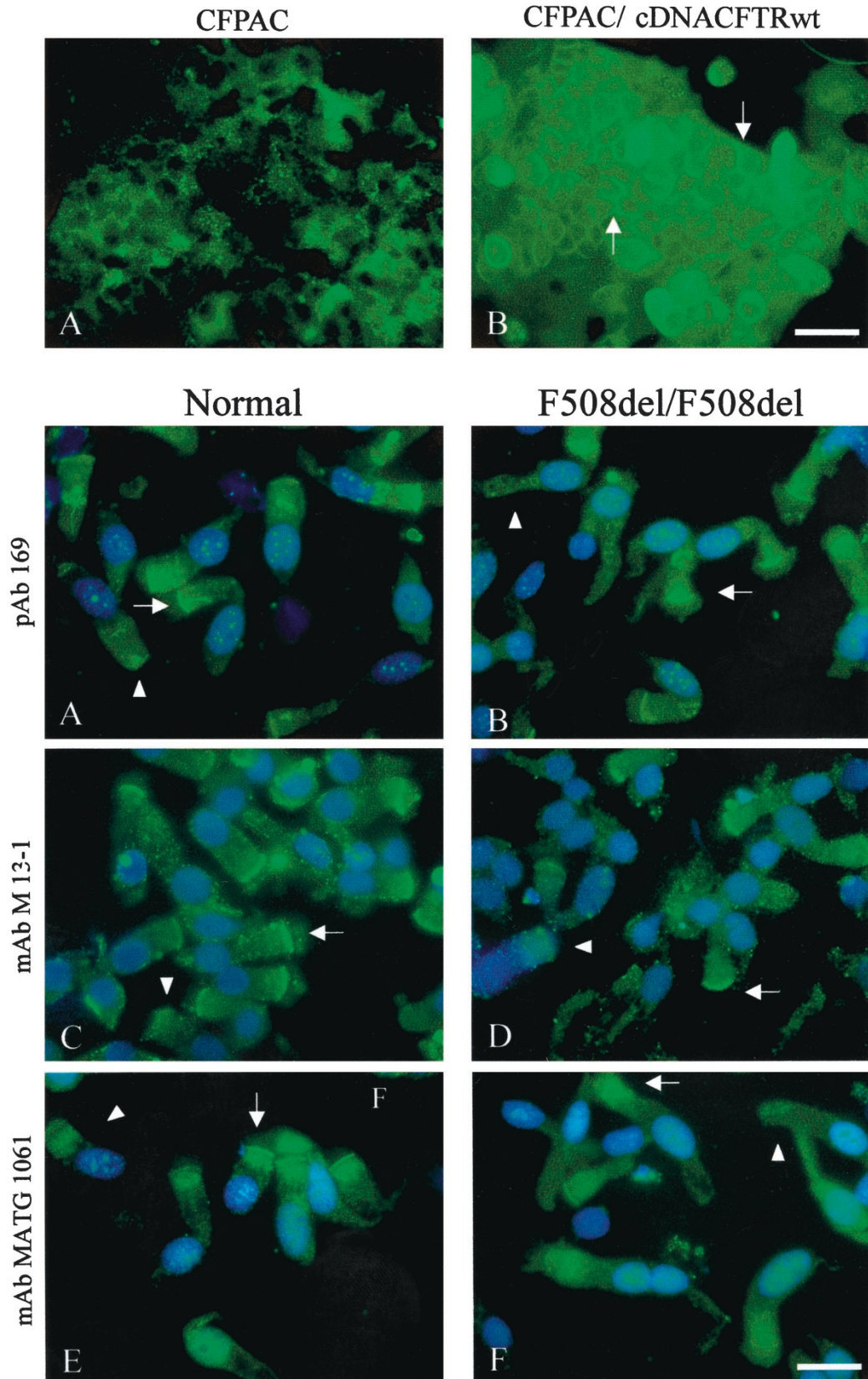
in Figure 5 clearly illustrate distinct distributions for the three groups. Indeed, a decreasing gradient in the proportion of TCE cells with CFTR in the AR is observed in the following order: non-CF subjects > F508del carriers > patients with CF.

## Discussion

Most of the current knowledge concerning localization of normal and mutant CFTR results from studies performed on heterologous expression systems (Cheng et al, 1990; Dalemans et al, 1991; Denning et al, 1992a; 1992b; Gregory et al, 1991; Lukacs et al, 1994), cultured human cells (Denning et al, 1992b; Engelhardt et al, 1992; McCray et al, 1993), or cell lines that suffered transformation (Demolombe et al, 1996; Engelhardt et al, 1992). Indeed, very few studies have dealt with this aspect in freshly isolated human airway cells from patients with CF. This is mainly because of the invasiveness and risk of most techniques aimed at collecting human cells (eg, biopsies), the small number of cells thus collected, or the limited number, poor quality, and nonrepresentative nature of samples resulting from surgery (such as nasal polypectomies or lung transplants). Brushing of the respiratory tract, however, a noninvasive method originally proposed for ciliary studies (Kelsen et al, 1992; Rutland and Cole, 1980; Rutland et al, 1982), allows the easy sampling of numerous, representative, well-preserved, and dissociated cells from the superficial mucosa (Bridges et al, 1991; Chapelin et al, 1996; Danel et al, 1996). The present study was thus performed on cell samples obtained by nasal brushings of F508del homozygous patients with CF, F508del carriers, and non-CF control subjects.

### Characterization of Cell Population in Nasal Brushing Samples

We characterized morphologically the cells recovered in nasal brushing samples to make sure that our method produced a representative sampling of cells. This analysis revealed that epithelial cells account for approximately 87% of all cells in the non-CF, carrier, and CF samples, with no significant differences among the three groups (Fig. 1C). With results similar to ours, other authors described the percentages of epithelial cells in nasal brushings of non-CF control subjects and patients with CF to be approximately 70% to 87% (Bridges et al, 1991), but this decreased to 65% in patients with chronic rhinitis (Chapelin et al, 1996). With regard to specific cell types in nasal brush samples, our results revealed a similar distribution among non-CF subjects, carriers, and patients with CF, ie, nonsignificant differences in all comparisons of specific cell types among the three groups analyzed (see Fig. 1C). The predominant subtype of epithelial cells was the ciliated cells (CC), comprising on average approximately 39% of all cells, followed by the nonciliated cells (NC) (23%), the basal cells (BC) (25%), and the nonidentified cells (NI) (8%). Other authors (Danel et al, 1996) found similar values, except



**Figure 2.** Top

Specificity of 169 anti-CFTR Ab. CFPAC-1 (A) and CFPAC-PLJ-6-CFTR (B) cells (see "Materials and Methods" section) were immunostained for CFTR with the 169 polyclonal Ab to detect CFTR. No CFTR is observed at the membrane of CFPAC cells (A). In CFPAC-PLJ-6-CFTR cells, however, this Ab clearly detects CFTR at the membrane (see *arrows*) demonstrating its specificity for CFTR. Similar results were obtained for both monoclonal antibodies M13-1 and MATG 1061 (data not shown). Bar = 40  $\mu$ m. *Figure 3 legend continued on next page.*

for CCs and BCs, which were determined to be approximately 57% and 8%, respectively (Chapelin et al, 1996). It is possible that the cells that we considered CCs are fewer in our study because we chose to include in this group only those in which cilia were distinctly observed (Fig. 4, O and R). Other authors consider preciliated cells to be ciliated as well. These are cells that, although not presenting cilia, have the same characteristic columnar shape and stain positively for tubulin at the apical membrane because of the presence of basal bodies (Chapelin et al, 1996). For BCs, the difference in percentages between our results (25%) and those of Chapelin et al (8%) may depend on the method used, ie, the type of brush used or the pressure applied on the brush by the operator. In our case, the brush was a hard, long-bristled interdental brush and may collect cells from deeper layers of the basal epithelium than the brushes used by other authors. Altogether, our results show that the populations of nasal cells recovered in CF and non-CF samples are similar and do not differ substantially from those previously described (Chapelin et al, 1996; Danel et al, 1996), thus validating the analysis of brush-obtained nasal cells.

#### Localization of CFTR in Nasal Brushing Cells

Three different and previously used (Brezillon et al, 1995; Crawford et al, 1991; Denning et al, 1992a; 1992b; Dupuit et al, 1995; Puchelle et al, 1992) anti-CFTR Abs, raised against different epitopes, were used in the present study to identify the intracellular localization of CFTR and F508del-CFTR in freshly obtained cells from the nasal epithelium. The specificity of the Abs was also confirmed under our immunocytochemistry protocol in CFPAC and CFPAC-PLJ-6-CFTR cell lines (Fig. 2) and also by double immunolabeling experiments (Fig. 4). By using each of those three Abs to immunolabel fresh nasal cells from non-CF subjects (Fig. 3), we observe that CFTR is in the AR of TCE cells, both ciliated and nonciliated (the latter comprising goblet cells). This confirms previous findings of immunocytochemical studies on fetal airways (Gaillard et al, 1994) and nasal polyps (Puchelle et al, 1992). However, it contradicts other immunocytochemical observations of the human bronchus, which were unable to detect CFTR in ciliated cells (perhaps using a less sensitive method), and restricting it to the submucosal glands (Engelhardt et al, 1992; 1994). We could also detect a faint intracellular CFTR-staining in basal cells, in accordance with previous findings (Brezillon et al, 1995).

#### Localization of F508del-CFTR in the Apical Region of Tall Columnar Epithelial Cells

The fact that we also detected F508del-CFTR in the AR of TCE cells from the nasal epithelium agrees with what was recently described by Kälin et al (1999), analyzing cryocuts of pseudostratified epithelium from CF and non-CF nasal polyps and to other reports analyzing biopsied human bronchial and nasal tissue by biochemical methods (Sarkadi et al, 1992; Zeitlin et al, 1992). However, it is contrary to a previous study, analyzing bronchial tissues from individuals with CF, which, however could have used a less sensitive method (Engelhardt et al, 1992). Our results also contradict most studies carried out in heterologous systems (Cheng et al, 1990; Dalemans et al, 1991; Denning et al, 1992a; 1992b; Gregory et al, 1991; Lukacs et al, 1994), cultured human cells (Engelhardt et al, 1992; Denning et al, 1992b; McCray et al, 1993), or immortalized cells (Demolombe et al, 1996; Engelhardt et al, 1992). However, it is most plausible that nonepithelial, and/or nonpolarized, cells lack some of the components needed for apical targeting.

We show, however, that in each sample (from non-CF subjects, carriers, and patients with CF), not all TCE cells have detectable CFTR in the AR. Although the method we used could not detect apparent differences in the localization of CFTR and F508del CFTR, the proportion of cells with CFTR in the AR is clearly distinct among samples from the three groups and decreases in the following order: non-CF subjects (56%) > carriers (42%) > patients with CF (22%). These differences were found to be statistically significant for the three Abs used (Table 2). It is, to our knowledge, the first time that a statistical treatment is applied to evaluate the presence of CFTR in the AR and that such a gradient is reported. However, there are descriptions in the literature that may account for this fact. Indeed, Kälin et al (1999) reported that their immunoblot analysis of polyps from patients with CF and non-CF subjects only gave positive results for a few specimens, which, besides the difficulties of the technique, may also account for an heterocellular distribution of CFTR in the AR. Although reporting normal CFTR protein levels in airways and intestinal epithelia of F508del homozygous patients by immunoblot analysis of microsomal membrane preparations, this technique involves many steps and thus introduces major quantification deviations. Another study with data from intestinal tissue analysis from the F508del CF mouse model also reports a faint signal obtained by immunoblot for the mature form of CFTR (French et al, 1996).

#### Figure 3.

Localization of CFTR in nasal brushing cells. Cells obtained in nasal brushing samples from non-CF individuals (panels A, C, and E) and patients with CF homozygous for F508del (B, D, and F) were used in the same protocol of immunocytochemistry as in Figure 2 with three different anti-CFTR antibodies: polyclonal 169 (A and B); monoclonal M13-1 (C and D); and monoclonal MATG 1061 (E and F). CFTR is detected by all three antibodies in the apical region (AR) of the tall columnar epithelial (TCE) cells, ciliated or not, present in these samples either from non-CF subjects or patients with CF (see arrows). Similar results were obtained for nasal brushing samples from carriers of F508del (not shown). Bar = 20  $\mu$ m.

**Table 1. Proportion of TCE Cells With CFTR in the Apical Region in the Three Groups Studied: Non-CF Controls, F508del Carriers, and CF Patients**

Ab	Statistical Parameters	Controls	F508del Carriers	CF Patients
169	mean ± SD	57% (±13.5%) (n = 11)	42% (±12.3%) (n = 20)	22% (±13.1%) (n = 12)
	median ± QD	56% (±9.6%) (n = 11)	44% (±3.9%) (n = 20)	22% (±10.7%) (n = 12)
M13-1	mean ± SD	57% (±7.3%) (n = 8)	39% (±9.6%) (n = 11)	24% (±12.0%) (n = 10)
	median ± QD	55% (±3.7%) (n = 8)	38% (±3.4%) (n = 11)	22% (±7.4%) (n = 10)
MATG1061	mean ± SD	61% (±8.4%) (n = 12)	43% (±9.4%) (n = 10)	27% (±18.0%) (n = 8)
	median ± QD	59% (±4.1%) (n = 12)	42% (±6.4%) (n = 10)	28% (±12.3%) (n = 8)

**F508del Mutation and Cystic Fibrosis Disease**

F508del-CFTR has been classified as a class II mutation (Welsh and Smith, 1993), following studies describing ER retention and consequent misprocessing as the major defect. Our results, evidencing a gradient for the proportion of TCE cells with CFTR in the AR in samples from non-CF subjects, carriers, and patients with CF, although supporting a trafficking defect associated with F508del-CFTR, also suggest that this process is leaky. Notwithstanding, decreased mRNA levels resulting from the F508del allele or decreased stability of the F508del-CFTR protein at the membrane could also explain our observations. By comparative quantitative analysis of CFTR and F508del-CFTR mRNA from the nasal epithelium of F508del-carriers, no significant reduction of F508del mRNA in comparison with the wt-CFTR product has been observed (Beck et al, 1999a ; 1999b; Dörk et al, 1994; Duarte et al, 1996; Trapnell et al, 1991). On the other hand, it was shown that F508del-CFTR protein is less stable than CFTR by an 8-fold difference when promoted to

accumulate in the plasma membrane of heterologous expression systems by lowering the temperature (Lukacs et al, 1993).

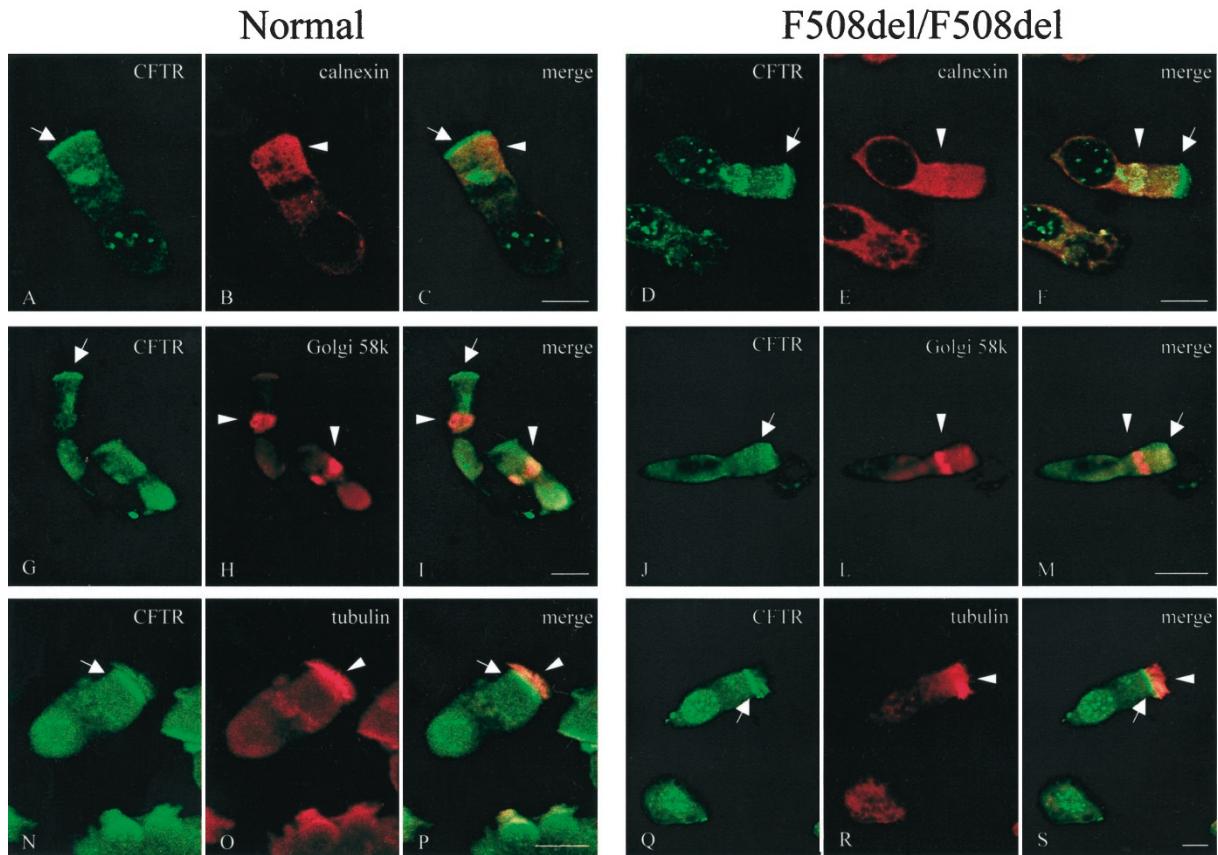
Considering the substantial evidence of increased nasal potential differences in patients with CF (Alton et al, 1987; Hay and Geddes, 1985; Hofmann et al, 1997; Knowles et al, 1981), it might be expected that in patients with CF the proportion of TCE cells with F508del-CFTR in the AR (22%) would be lower. The sensitivity of the nasal potential difference assay for residual Cl<sup>-</sup> secretory activity, however, remains to be determined (Pilewski and Frizzell, 1999). Functional analysis of freshly isolated nasal epithelial cells from CF F508del patients and non-CF individuals was described in another study and evidenced deficient cAMP-directed Cl<sup>-</sup> secretion in CF (Stern et al, 1995). However, again a semi-quantitative method was used. A more sensitive approach used to study native tissue, however, was applied to the analysis of rectal biopsies from CF F508del patients, where a residual Cl<sup>-</sup> secretion of 3% was described (Mall et al, 2000; Veeze et al, 1994).

**Table 2. Significance Tests for Comparisons in the Proportion of TCE Cells with CFTR in the AR for the Three Groups Studied**

Ab	Comparison	Test	p value	Significance <sup>B</sup>
169	Controls vs. Patients	student <i>t</i>	5.4 ± 10 <sup>-7</sup>	*
	Controls vs. Carriers	student <i>t</i>	1.8 ± 10 <sup>-3</sup>	*
	Patients vs. Carriers	student <i>t</i>	1.5 ± 10 <sup>-4</sup>	*
	All 3 groups	ANOVA	1.0 ± 10 <sup>-7</sup>	*
M13-1	Controls vs. Patients	student <i>t</i>	4.3 ± 10 <sup>-7</sup>	*
	Controls vs. Carriers	student <i>t</i>	9.1 ± 10 <sup>-5</sup>	*
	Patients vs. Carriers	student <i>t</i>	4.2 ± 10 <sup>-3</sup>	*
	All 3 groups	ANOVA	1.2 ± 10 <sup>-7</sup>	*
MATG 1061	Controls vs. Patients	Mann-Whitney <sup>a</sup>	3.9 ± 10 <sup>-4</sup>	*
	Controls vs. Carriers	student <i>t</i>	8.0 ± 10 <sup>-5</sup>	*
	Patients vs. Carriers	student <i>t</i>	2.7 ± 10 <sup>-2</sup>	*
	All 3 groups	Kruskal-Wallis <sup>a</sup>	6.5 ± 10 <sup>-5</sup>	*

\* Asterisk refers to differences significant at *p* < 0.05.

<sup>a</sup> Parametric tests not applied, because the hypothesis of equal variances was rejected (see "Materials and Methods" section).



**Figure 4.**

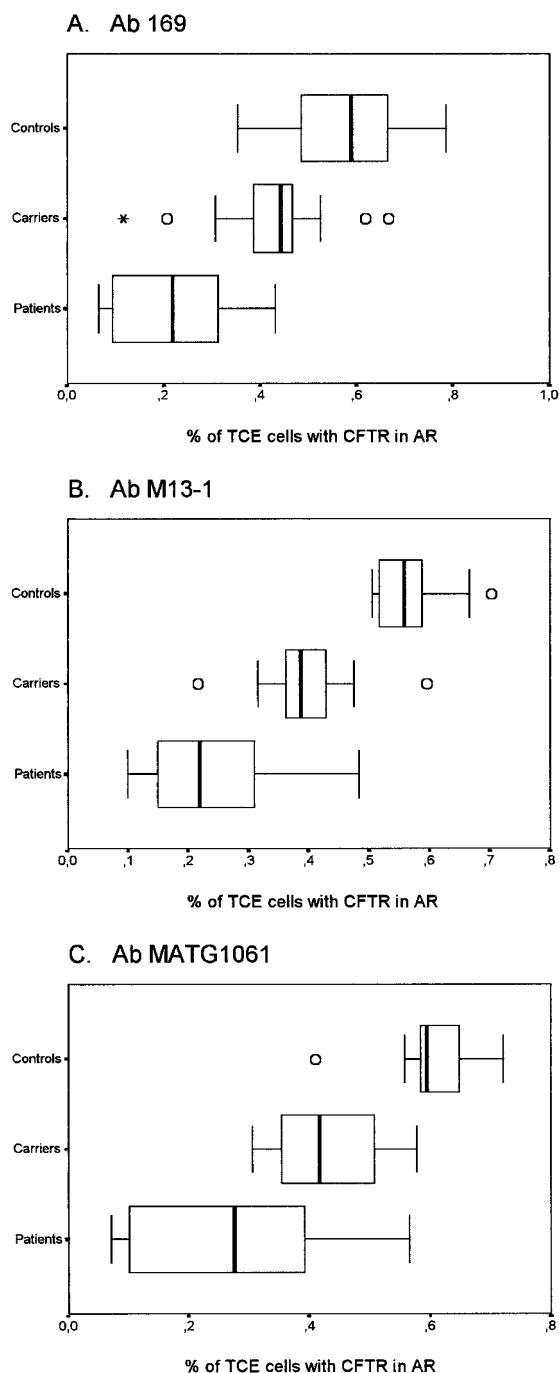
Double labeling of CFTR, calnexin, p58, and tubulin. Double immuno-labelings of fresh cells from nasal brushings were carried out (see "Materials and Methods" section) with polyclonal anti-CFTR 169 Ab (in green, see arrows in panels A-C, D-F, G-I, J-M, N-P, and Q-S) and with monoclonal anti-calnexin Ab (in red) staining mostly the ER (arrowheads in B-C and E-F); anti-p58 (in red) staining the Golgi (arrowheads in H-I and L-M); and monoclonal anti-tubulin Abs (also in red) staining the cytoskeleton, microtubules (not seen), and cilia (arrowheads in O-P and R-S). Neither the anti-calnexin Ab (B and E) nor anti-p58 (H and L) stain the surface of TCE cells as does the anti-CFTR 169 Ab (A and D). Some overlap occurs between labeling of anti-CFTR and anti-calnexin (C and E), particularly for the F508del cells (E) and also between anti-CFTR and anti-p58 (I and M). Anti-tubulin clearly stains the cilia of TCE cells from non-CF subjects (O) and from patients with CF (R), but little overlap is observed with anti-CFTR (P and S). Bar = 10  $\mu\text{m}$  in all micrographs.

Although similar studies have not been performed in human airway tissue, except for nasal polyps (Kunzelmann et al, 1995), evidence that some F508del-CFTR may become functionally expressed in the apical membrane comes from studies in mice by Kelley et al (1997). These authors demonstrated that the combination of forskolin and milrinone hyperpolarizes the nasal epithelium of F508del mice, but not of CFTR ( $-/-$ ) mice, which is indicative that some mutant protein is in the apical region and may induce a  $\text{Cl}^-$  secretory response.

The most plausible explanation for the apparent conflict between our results and the existing functional data (nasal PDs) are that F508del-CFTR is not equivalent to CFTR in terms of its function as a  $\text{Cl}^-$  channel, as evidenced by several groups. One study (Dalemans et al, 1991) reports that F508del-CFTR exhibits an open probability three to four times lower than CFTR, and another describes reduced sensitivity to cAMP (Drumm et al, 1991). Most laboratories now agree that F508del-CFTR exhibits a reduced open probability in comparison to CFTR (Pilewski and Frizzell, 1999). Therefore, besides class II, F508del should also be considered as a class IV mutation.

Our immunocytochemical data do not exclude the localization of both CFTR and F508del-CFTR in subapical membrane vesicles, as described in studies using the powerful immunogold transmission electronic microscopy technique (Puchelle et al, 1992; Webster et al, 1994) and in several biochemical and functional studies (Biversi and Verkman, 1994; Bradbury and Bridges, 1994; Lukacs et al, 1993; 1997). Indeed, we observe non-CFTR labeling as a broad area in the AR, which is consistent with both apical and subapical localizations.

It is thus possible that both CFTR and F508del-CFTR lie mostly in the subapical region and that, upon stimulation (eg, by cAMP) CFTR, but not F508del-CFTR, undergoes exocytosis to the apical membrane, as first suggested by Bradbury et al (1992) and also by other authors (Biversi et al, 1996; Prince et al, 1994; Takahashi et al, 1996; Tousson et al, 1996). The fact that a number of other studies reported apparently contradictory observations (Dho et al, 1993; Dunn et al, 1994; Hug et al, 1997), provides evidence that the issue of CFTR and vesicular trafficking is still an open



**Figure 5.** Statistical analysis of tall columnar epithelial (TCE) cells with CFTR in the apical region (AR). Based on percentages of TCE cells with CFTR in the AR relative to total TCE cells in samples from nasal brushings (Table 1) for non-CF subjects, carriers, and patients with CF, graphs A, B, and C were constructed corresponding to CFTR immunodetection with three different anti-CFTR Abs: 169 (A), M13-1 (B), and MATG 1061 (C), respectively. Graphs clearly display the differences in distributions for the proportion of TCE cells with CFTR in the AR for the three groups.

question. Many of those studies were carried out in non-native systems and thus argue for examining this question in native physiological tissues that possess all necessary elements required for acute regulation of membrane trafficking.

In conclusion, the present results are consistent with a trafficking defect for F508del-CFTR, which could occur at two levels, namely, 1) ER retention and degradation, and/or 2) failure in recruitment from sub-apical vesicles to the apical membrane. Other alterations, however such as decreased stability at the membrane and impaired channel properties, can also contribute to the disease phenotype caused by the F508del mutation. Solid evidence for or against occurrence of residual Cl<sup>-</sup> secretion in fresh airway tissue from patients with CF who are homozygous for F508del would be of great help in clarifying the CF airway disease defect(s) associated with this mutation. Pharmacological attempts aimed at circumventing those alterations, such as described for the CF F508del mouse (Kelley et al, 1997), may be of therapeutic value for the treatment of F508del homozygous patients with CF.

## Materials and Methods

### Individuals and Genotypes

Cells from nasal brushings were obtained from patients with CF homozygous for F508del (*n* = 12), carriers of the F508del mutation (*n* = 20), and healthy individuals (*n* = 12) with no clinical signs of CF nor F508del mutation, as determined by genomic DNA analysis. Genotypes of all individuals studied were determined as described before (Duarte et al, 1996).

### Nasal Brushings and Recovery of Cells

After informed consent, nasal brushings were performed as described before (Beck et al, 1999b). Briefly, an interdental brush with 2.5 to 3 mm bristles (Paro-Isola, Thalwil, Switzerland) was used to scrape along the tip of the inferior turbinate and the adjacent lateral nasal wall. Brushes with cells were immediately placed into phosphate-buffered saline (PBS) pH 7.4 at room temperature. Cellular material was removed from the brush by flicking it up and down inside a disposable P200 pipette tip. Cells were then washed in PBS and centrifuged at 3,000 rpm in a Picofuge (Stratagene, La Jolla, California) for 4 minutes, gently resuspended from pellets and fixed in 3.7% (v/v) formaldehyde, 3.7% (w/v) sucrose in PBS for 30 minutes at 4° C. After centrifugation under the same conditions, cells were resuspended in cold PBS and kept at 4° C until analysis. For adherence to silane-coated glass slides (Sigma Chemical Company, St. Louis, Missouri), cells were centrifuged for 5 minutes at 1,000 rpm in a Cytospin 3 centrifuge (Shandon, Life Sciences International Ltd, Cheshire, United Kingdom). One slide per sample was taken for cytologic examination, and the others were used for immunocytochemical analysis.

### Cell Lines

CFPAC-1, a pancreatic epithelioid cell line obtained from patients with CF (Schoumacher et al, 1990), and CFPAC-PLJ-6-CFTR cell line, obtained by retroviral



stable transfection of CFPAC-1 with the full-length, wild-type *CFTR* cDNA (Drumm et al, 1990), were used as controls of our immunocytochemical protocol. Both cell lines were cultivated as previously described, after seeding onto 8-well chamber slides (Nalgenunc International, Rochester, New York) at a density of  $10^4$  cells/well and analyzed 2 days after.

### **Evaluation of Cellular Types Recovered by Nasal Brushing**

The freshly isolated human cells recovered from nasal brushings and spread on silane glass slides were stained by the May-Grünwald-Giemsa (MGG) method (Dacie and Lewis, 1984). After 5 minutes fixing in methanol, slides were immersed for 5 minutes in May-Grünwald's standard stain (Fluka Chemie AG, Buchs, Switzerland), freshly diluted with an equal volume of phosphate buffer pH 6.8, and then, without washing, immersed for 10 to 15 minutes in Giemsa stain (Merck Diagnostica, Darmstadt, Germany) diluted with nine volumes of phosphate buffer pH 6.8. After 3 to 4 rapid washes in phosphate buffer pH 6.8 and 2 to 5 minutes in water, slides were mounted with Entellan (Merck), covered with glass coverslips, and dried for at least 1 hour before analysis. Samples on slides were evaluated for cell differential count and morphology on a conventional light microscope (Zeiss, Jena, Germany) at a  $100\times$  magnification under oil immersion. Epithelial cells were classified into three major categories (ciliated, nonciliated, and basal) on the basis of described criteria (Danel et al, 1996). Ciliated cells had tall columnar shapes with distinct cilia, nonciliated cells, including secretory goblet cells, had similar shape but no cilia, and basal cells were smaller, with dense, round nuclei, strongly stained cytoplasm, and a high nuclear-to-cytoplasmic ratio. Inflammatory cells, ie, lymphocytes, neutrophils, eosinophils, and macrophages, were all included in the same group. Other cells that did not meet any of these criteria were called "not identified" (NI).

### **Antibodies**

All Abs were diluted in 0.5% (w/v) BSA in PBS. The following anti-CFTR Abs, previously shown to recognize CFTR, were used: polyclonal 169, raised against the peptide comprising amino acids (aa) 724–746, in exon 13 of the R-domain of CFTR (Crawford et al, 1991), diluted 1:100; monoclonal M13–1 (Genzyme, Cambridge, Massachusetts), against aa 729–736 of the R-domain (Denning et al, 1992a; 1992b; Gregory et al, 1990), 1:20; and monoclonal MATG 1061 (Transgène, Strasbourg, France) (Brézillon et al, 1995; Puchelle et al, 1992), against aa 503–515 of NBF1, 1:20. The epithelial nature of cells was determined by using Ab anti-cytokeratin (Clones AE1/AE3; Boehringer, Mannheim, Germany), 1:1500. Ciliary structures were tubulin-stained with a 1:1 mixture of two monoclonal Abs against  $\alpha$ - and  $\beta$ -tubulin (Amersham Pharmacia Biotech AB, Uppsala, Sweden), 1:100. Monoclonal anti-calnexin Ab AF8 (Hochstenbach et al, 1992), 1:60,

was used to stain the endoplasmic reticulum. Monoclonal anti-p58 Ab (Sigma), 1:60, was used to stain the Golgi apparatus (Bloom and Brashear, 1989). Secondary Abs were FITC-conjugated, anti-rabbit IgG (Amersham), 1:50 (for 169 Ab), and FITC-conjugated anti-mouse IgG (Boehringer), 1:80 (for mAbs M13–1 and MATG 1061); TRITC-conjugated, anti-mouse IgG (Sigma), 1:600 (for anti-tubulin, anti-calnexin, and anti-p58 Abs).

### **Immunocytochemistry**

Unless otherwise indicated, all incubations were kept at room temperature and all solutions in PBS. After fixation (see above), cells on slides were rinsed for 5 minutes with cold PBS and incubated in methanol for 5 minutes at  $-20^\circ\text{C}$ , followed by two washes with PBS and incubation in 0.25% (v/v) Triton X-100 for 10 minutes, for cell permeabilization. Nonspecific staining was prevented by blocking with 1% (w/v) BSA for 30 minutes and incubation with one of the anti-CFTR Abs (169, M13–1, or MATG) overnight at  $4^\circ\text{C}$ . After three 5-minute washes with PBS, cells were incubated with the respective secondary Ab for 30 minutes and washed as above. For double labeling experiments, fixing, permeabilization, and blocking were performed as above, followed by incubation with the anti-CFTR 169 Ab overnight at  $4^\circ\text{C}$ , and after three 5-minute washes, with the other primary Ab (anti-calnexin, anti-tubulins, or anti-p58) for 1 hour at  $37^\circ\text{C}$ , followed by the same washing procedure. Reactions with the two secondary antibodies were carried out consecutively for 45 minutes each, with three 10-minute washes with PBS in between. Slides were mounted with Vectashield (Vector Laboratories, Inc., Burlingame, California), containing DAPI (4, 6-diamino-2-phenylindole, Sigma) for nuclei staining, and covered with a glass coverslip. Immunofluorescence staining was observed and collected on an Axioskop fluorescence microscope (Zeiss) with the Power Gene 810/Probe and CGH software system (PSI, Chester, United Kingdom). Confocal images were obtained in a MRC-600 confocal imaging system (Bio-Rad, Hercules, California).

### **Statistical Analysis**

Data are summarised by the mean  $\pm$  standard deviation (SD) and by median  $\pm$  quartile deviation (QD). The hypothesis of equal variances between the three groups analyzed (non-CF subjects, F508del-carriers, and patients with CF) was tested using the Levene's test for each analysis, and there was no evidence to reject it ( $p > 0.05$ ), except between distributions relative to patients and non-CF subjects with Ab MATG 1061 ( $p = 0.027$ ). Statistical significance comparisons for samples with equal variances were made using the parametric Student's *t* test for two unpaired samples and the parametric one-way ANOVA for three independent grouped samples (Sokal and Rohlf, 1981). For samples with unequal variances, we used the nonparametric Mann-Whitney *U* test (Wilcoxon) for

two independent samples and the nonparametric Kruskal-Wallis-test, for three independent grouped samples (Sokal and Rohlf, 1981). Coefficients with a  $p$  value less than 0.05 were considered to be statistically significant. The SPSS for Windows software (SPSS Inc., Chicago, Illinois) was used for all statistical calculations.

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