Author's response to reviews

Title: In vitro prediction of stop-codon suppression by intravenous gentamycin in patients with cystic fibrosis; a pilot study.

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Author's response to reviews:

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Dear Editor,

Thank you for your letter of November 20, concerning the possible resubmission of our manuscript. The manuscript has been revised in light of the two reviewers' helpful comments and suggestions. We have made the formatting changes requested in your letter and included in the abstract the number of the trial registration.

Please find enclosed the revised version of our manuscript, and the point-by-point reply to the comments of each of the reviewers, as requested.

Yours sincerely,

I Sermet-Gaudelus

Point by point response to reviewers

Reviewer 1

1. Comment 1. Reviewer 1. A drawback of the study is the small size of the groups. This results in a weak significance for the effects in the Y122X group.

We agree with the reviewer that the size of the sample of patients is small. However we emphasize that the Y122X mutation is a rare mutation mainly found in the Reunion Island. Considering the strong readthrough efficiency directed by this mutation, we decided to focus on patients homozygous for this mutation. Only 11 patients were homozygous for this mutation among the centers of this study, the majority of those, living in the Reunion Island. This low sample of patients who might be enrolled in the study therefore explains the low sample of the study.

NPD measurements were the main endpoint for our study. Changes in [increment]chloride-free isoproterenol > |-2| mV were considered significant. We chose this cutoff based on our NPD reproducibility data: only 2% of the [increment]chloride-free isoproterenol readings for the same subject had a variation of more than 2 mV, when this subject had at the first test a response < - 2 mV. At least 6 patients were required to detect a significant change > |-2| mV in response to chloride-free isoproterenol solution, with 80 percent power and an alpha error of 5 percent. Therefore, we considered that including 9 patients in the
Y122X group was sufficient for statistical significance.

2. Comment 1. Reviewer 1. There is also the question that the Y122X group does not react homogeneously. Does this imply that other factors besides the genotype are important for the success of the treatment? Among the 9 Y122X patients, 2 had no response to gentamicin treatment. This is similar to the results described with PTC124 in patients with the W1282X mutation (Batsheva Kerem et al. Symposium Session 4.2 NACFF Denver 2006). The variety of different mechanisms that modulate readthrough activity may explain this lack of efficacy.

1. The gentamicin in contact with the target cells is of critical importance because suppression of stop mutations is dose dependent. (Barton Davies et al. J Clin Invest 1999). In our study, serum and sputum gentamicin concentrations peaked at higher levels in patients for whom CFTR was detected at the end of the study: respectively 25.8(2) ug/mL versus 20.6(3.3) ug/mL; p=0.05 and 2.4(1.4) ug/mL versus 1.7(1.3) ug/mL, NS). Interpatient variability in gentamicin could account for this difference. These results are consistent with data from mdx mice showing that mistranslation does not occur below a certain translation and that the concentration of full-length CFTR protein after incubation with gentamicin parallels the increase in the antibiotic dose (Barton Davies et al.). The low gentamicin dose in Clancy's study (Clancy et al, Am J Resp Crit Care Med 2001) may explain the absence of clinical effect observed there: gentamicin dosing was adjusted to achieve peak serum levels of 8-10 ug/ml with a 2.5 mg/kg three times a day administration, while we aimed for peak concentrations of 20-40 ug/ml with a 10 mg/kg once a day administration, a dosage regimen known to achieve higher serum concentrations in CF patients without toxic effects. We therefore hypothesize that a critical concentration is needed to obtain significant readthrough. But also that, above a certain threshold, the misread rate may be too high for functional protein synthesis, as assessed by the antibacterial activity. We propose that future clinical studies measure the gentamicin concentration in contact with the target cells to identify the concentration that best promotes useful preferential misreads. We have included this discussion in the text (page 11, line 23 and followings).

2. Genetic, not CFTR-related, modifying factors might also cause variations in gentamicin-induced-readthrough. One might hypothetize that some genes might regulate the "sensitivity" to gentamicin induced effect, i.e. the amount of local concentration in contact to the target cells necessary to induce efficient readthrough. The modulation of the nonsense-mediated mRNA decay (NMD) pathway might also modify the readthrough. The group of B Kerem found a considerable variability in the level of CFTR nonsense transcript among the patients carrying the W1282X mutation mutation (Batsheva Kerem et al. Symposium Session 4.2 NACFF Denver 2006). A significant reduction in basal potential difference and or a significant response to chloride-free isoproterenol solution was found in all the patients with relatively high levels of CFTR nonsense transcripts. No correction of the CFTR electrophysiological abnormalities after gentamicin treatment was evidenced in the patients with markedly reduced levels. Altogether, these results suggest that the level of regulation of the NMD pathway may be a modifying factor in the readthrough of premature stop codon, in response to gentamicin and therefore might explain the presence of both "reponders and non responders" in patients carrying the same mutation.

3. Comment 3. Reviewer 1. The practical consequences of the study seem to be limited, in that gentamycin is not effective for all class I mutations..., but only for a small subgroup...., and may be not even for all patients of this subgroup.

From our point of view, this study provides important data concerning systemic effect of gentamicin-induced readthrough. It is the first study demonstrating that pharmacological suppression of CFTR premature stop mutations can restore adequate levels of functional protein and therefore improve the clinical status of the patients. Moreover, this multidisciplinary pharmacogenetic approach demonstrates a correlation between the evaluation of the readthrough efficiency first in vitro by a dual reporter gene assay, and then in the CF patients by clinical, functional, and immunological parameters. This suggests that trials of patients with premature stop codon diseases should be preceded by cell assays studies to identify the patients most likely to benefit from a clinical trial with gentamicin.
Comment 3. Reviewer 2. The figure legends and numbers do not correspond. This has been changed.

Comment 4. Reviewer 2. In the sweat chloride figure and the NPD figures, representative data from normal controls should be included to give a better idea of the magnitude of the response. Data of normal controls have been included. These data were previously published from our control cohort. Sermet-Gaudelus et al. Am J Respir Crit Care Med 2005;171(9):1026-31

Comment 5. Reviewer 2. In the figure depicting immunostaining of CFTR, which antibody is used. Immunostaining from normal control cells is also needed for comparison of the figure. The 24-1 Antibody was used. Data from normal control (NPD and immunostaining) has been given in a separate figure.

Comment 6. Reviewer 2. No time scale is given for NPD tracings. A time scale has been given.