Validation of the refined thresholds in an additional independent cohort

The effectiveness of the refined thresholds in identifying affected 4qA/4qA were tested on an additional independent cohort of 315 subjects available at our laboratory. After filtering this cohort for genotype, 152 4qA/4qA subjects out of 315 were selected. The application of the new CpG6 and CpG3 thresholds (0.8493 and 0.2222, respectively) to this sub-group allowed classifying 68 patients (out of 152) as FSHD, which were consistent with the presence of disease-associated molecular signatures. The remaining 84 showed methylation levels above the new thresholds. Among them, 77 out of 84 were unaffected subjects with *D4Z4* size>10RU and were therefore correctly classified as non-FSHD whereas seven subjects were Non-Concordant-Negative (NCN). The performance metrics obtained by applying the refined thresholds to the independent cohort are summarized in Figure 1.

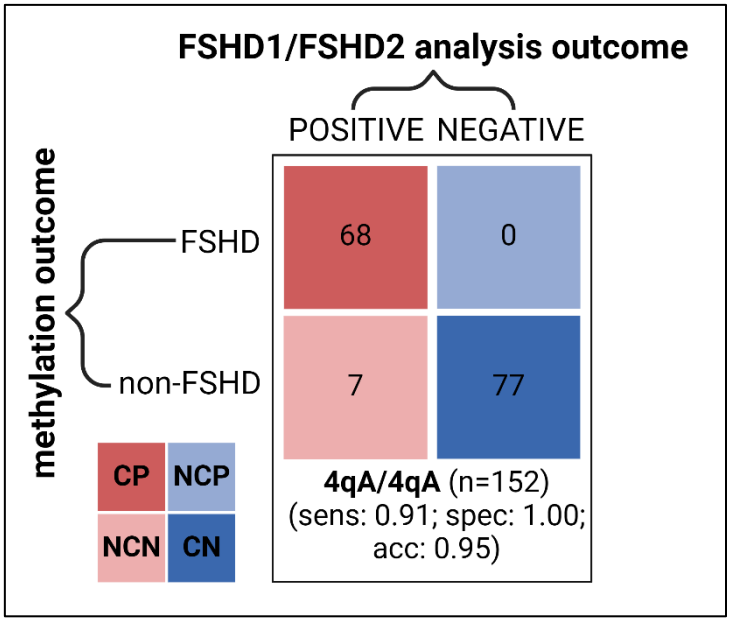

Figure 1. Performance metrics obtained on the 152 4qA/4qA subjects of the independent cohort of 315 subjects.

Despite carrying a *D4Z4* shorter than 10 RU, the methylation levels of these subjects were apparently not suggestive of FSHD. In particular, five patients carried a 4qA of 5RU and the remaining two had 6 and 7RU, respectively. Four out of seven NCN subjects had a positive family history of FSHD. Unfortunately, no clinical data were available for most of them. The variable penetrance and expressivity often observed in FSHD families could explain borderline methylation levels showed by these patients.