Title: *PHOX2B* Non-Polyalanine Repeat Expansion Mutations in Congenital Central Hypoventilation Syndrome: Advancing Understanding of Phenotype by Mutation Type through Industry-Academic Medicine Collaboration

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Rationale:
Congenital central hypoventilation syndrome (CCHS) is a rare neurocristopathy caused by mutations in *PHOX2B*, a gene expressed in early embryology of the autonomic nervous system. We previously described a *PHOX2B* genotype-CCHS phenotype relationship for heterozygous polyalanine repeat expansion mutations (PARMs; which cause ~90% of CCHS), allowing risk assessment for Hirschsprung disease, cardiac sinus pauses, and neural crest tumors, and predictions for artificial respiratory needs. In the more rare non-PARMs (NPARMs; causing ~10% of CCHS), phenotypes associated with specific genotypes remains unclear. This study aims to expand knowledge of NPARMs and associated phenotypes with a unique collaboration between commercial and academic laboratories.

Methods:
PHOX2B clinical testing was performed by Ambry Genetics, Rush University, and Ann & Robert H. Lurie Children's Hospital of Chicago between 2003 and 2016. Mutations were stratified into frameshift, missense, nonsense, stop codon alterations, and other. Phenotypic information was extracted from test requisition forms, referrals, and the International CCHS REDCap Registry. Mutated protein size and protein change were determined through Mutalyzer (https://www.mutalyzer.nl/).

Results:
102 NPARM cases were identified (20 previously published), along with 75 published NPARM cases from other laboratories, (total 177 NPARM cases, 97 unique variants). Maximum and minimum NPARM protein size was 364 and 4 amino acids, respectively (normal 314). All frameshift mutations in exon 1 and 2 led to protein truncations, though exon 3 contained NPARMs causing both truncations and elongations. Within and after the 20-alanine region of exon 3, nearly all mutations resulted in elongated proteins. Frequency of phenotypic features is presented in Table 1. Neuroblastomas were the most common tumors, and strongly associated with frameshift (63%) and nonsense mutations (50%). Ganglioneuroblastomas and ganglioneuromas were associated with missense (11%), frameshift (7%), stop codon (50%) and missense mutations (22%).

Conclusions:
Through collaboration between academic and commercial laboratories, this study is the largest cohort of NPARMs and associated phenotype data, offering opportunity for anticipatory management by mutation type. Phenotypic frequencies are likely underestimated due to

PHOX2B analysis often preceding diagnosis of many clinical features, so follow-up from laboratories is essential to characterize the full phenotype for each child and each mutation type. Continued investigation through family, functional, or segregation studies will allow further elucidation of variable expression and penetrance within families for a subset of NPARMs, as well as the pathogenic mechanisms involved with these mutation types.

Table 1. *PHOX2B* Non-Polyalanine Repeat Expansion Mutations (NPARMs) by Type and by Accessible Phenotypic Features

Mutation Type	# Mutations	CCHS (includes apnea or hypoventilation) (%)	Hirschsprung (%)	Neural crest tumor (%)	Cardiac pacemaker (%)	Cardio-respiratory arrest (%)	Seizures (%)
Nonsense	10	100	50	50	0	---	---
Missense	47	100	80	72	22	13	55
Frameshift	105	100	88	77	0	25	55
Stop codon	7	100	100	100	---	---	---
Other	8	75	67	0	0	0	0

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