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State of the Art: Sleep Disordered Breathing in Children

Congenital Central Hypoventilation Syndrome From Past to Future: Model for Translational and Transitional Autonomic Medicine

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Summary. The modern story of CCHS began in 1970 with the first description by Mellins et al., came most visibly to the public eye with the ATS Statement in 1999, and continues with increasingly fast paced advances in genetics. Affected individuals have diffuse autonomic nervous system dysregulation (ANSD). The paired-like homeobox gene *PHOX2B* is the disease-defining gene for CCHS; a mutation in the *PHOX2B* gene is requisite to the diagnosis of CCHS. Approximately 90% of individuals with the CCHS phenotype will be heterozygous for a polyalanine repeat expansion mutation (PARM); the normal allele will have 20 alanines and the affected allele will have 24–33 alanines (genotypes 20/24–20/33). The remaining ~10% of individuals with CCHS will have a non-PARM (NPARM), in the *PHOX2B* gene; these will be missense, nonsense, or frameshift. CCHS and *PHOX2B* are inherited in an autosomal dominant manner with a stable mutation. Approximately 8% of parents of a CCHS proband will be mosaic for the *PHOX2B* mutation. A growing number of cases of CCHS are identified after the newborn period, with presentation from infancy into adulthood. An improved understanding of the molecular basis of the *PHOX2B* mutations and of the *PHOX2B* genotype/CCHS phenotype relationship will allow physicians to anticipate the clinical phenotype for each affected individual. To best convey the remarkable history of CCHS, and to describe the value of recognizing CCHS as a model for translational and transitional autonomic medicine, we present this review article in the format of a chronological story, from 1970 to the present day. **Pediatr Pulmonol. 2009; 44:521–535.**

Key words: congenital central hypoventilation syndrome; autonomic dysregulation; Hirschsprung disease; neuroblastoma.

"Apparently rare cases are worth studying, not because they are rare, but because they provide an opportunity to unravel an important homeostatic mechanism of disease present in all of us but not so apparent except in those missing some important protective mechanism."

Robert B. Mellins, M.D. 2007

This article is one in a series of articles on Sleep Disordered Breathing in Children. This series will span upcoming issues of Pediatric Pulmonology.

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CONGENITAL CENTRAL HYPOVENTILATION SYNDROME (CCHS) FROM THE BEGINNING...

The modern story of CCHS began in 1970 with the seminal case report by Dr. Robert Mellins and coworkers, entitled "Failure of Automatic Control of Ventilation,"[1] came most visibly to the public eye with the American Thoracic Society (ATS) Statement on CCHS in 1999,[2] and continues with increasingly fast paced advances in genetics.[3] Though the hallmark of CCHS is the respiratory dysregulation, CCHS is far more complex than a simple orphan disorder of respiratory control. Individuals with CCHS are diagnosed in the absence of primary lung, cardiac, or neuromuscular disease or an identifiable brainstem lesion that might account for the entire phenotype inclusive of the autonomic nervous system dysregulation (ANSD). Individuals with CCHS characteristically have diminutive tidal volumes and monotonous respiratory rates awake and asleep,[2] though more profound alveolar hypoventilation occurs primarily during sleep. As a result of hypoventilation these individuals become hypoxemic and hypercarbic but lack the normal ventilation and arousal responses to these endogenous challenges during sleep,[2] and the perception of asphyxia during wakefulness with and without exertion.[2] Nonetheless, they maintain the ability to consciously alter the rate and depth of breathing. Conditions associated with CCHS include Hirschsprung Disease and tumors of neural crest origin, in addition to a spectrum of symptoms compatible with ANSD including diminished heart rate variability and transient abrupt asystoles, decreased pupillary light response, esophageal dysmotility, breath-holding spells, reduced basal body temperature, sporadic profuse sweating, lack of perception to dyspnea, altered perception of anxiety, and lack of physiologic responsiveness to the challenges of exercise and environmental stressors.[2,4–17]

CCHS is characteristically diagnosed in the newborn period, yet we now know that cases can be diagnosed later in infancy and childhood[18–21] as well as adulthood.[20,22–28] To best convey the remarkable history of CCHS, and to describe the value of recognizing CCHS as a model for translational (bench to bedside) and transitional (childhood to adulthood) autonomic medicine, we present this review article in the format of a chronological story, from 1970 to the present day.

CHAPTER ONE: THE SEMINAL CASE REPORTS (APPROXIMATELY 1970–1978)

Dr. Robert Mellins and coworkers described the first child with characteristic CCHS in 1970.[1] The subject of that case report was an infant boy who was cyanotic on nursery admission, during sleep, and with feeding. He had an attenuated ventilatory response to inhalation of 0.04 F_ICO_2 and no change in mental state despite pCO_2 values of 120–130 mmHg. His body temperature was reduced, he had esophageal dysmotility, and he had respiratory deterioration with an upper respiratory infection. The next major milestone in describing CCHS came in 1978 with Dr. Gabriel Haddad's publication entitled "Congenital Failure of Automatic Control of Ventilation, GI Motility, and Heart Rate."[29] Dr. Haddad and coworkers made the association between CCHS, Hirschsprung Disease and tumors of neural crest origin. They described an infant girl who was cyanotic at 3 hr of life, with improved ventilation in rapid eye movement (REM) sleep. She had an attenuated ventilatory response to inhalation of 0.02 F_ICO_2, lack of heart rate variability, and generalized hypotonia. She also had Hirschsprung Disease and multiple ganglioneuroblastomas. By describing the extended phenotype in two sisters, the publication provided the first suggestion that CCHS is familial. Between 1970 and 1978 fewer than 25 single or small cohort case reports on CCHS were written. Though recognized, CCHS was still considered an exceedingly rare disorder.

CHAPTER TWO: GROWING INTEREST IN CCHS, MOSTLY CASE REPORTS, HINTS OF FUTURE TERM "ANSD," AND FIRST PEDIATRIC SLEEP LABORATORIES (APPROXIMATELY 1979–1991)

During this time, there was a growing interest in CCHS with an increasing number of published case reports, yet totaling fewer than 100 cases. In careful reading of those case reports a sense of what would later be termed "autonomic nervous system dysregulation" was described with variable symptoms in varying organ systems. Further, the first pediatric sleep laboratories were being developed commensurate with a growing awareness of obstructive sleep apnea in the general pediatric population. The increasing sophistication of the pediatric sleep laboratory made it possible to study children with CCHS in a physiologically comprehensive manner.

CHAPTER THREE: EXPANDED STUDY SIZES, DEVELOPMENT OF CCHS CENTERS OF EXCELLENCE, AND SEGREGATION ANALYSIS GENETIC STUDY (APPROXIMATELY 1992–1995)

In 1992 complete description of the first large, 32 cases, cohort was published.[5] Internationally, there was an emergence of Centers of Excellence for the study of CCHS—with recognition that rare disease cohorts need to be consolidated to maximally learn from the children and to optimally improve the care of the affected children. The ability to study large cohorts of children with CCHS at these Centers led to the major advances that followed.

Segregation analysis established an important link between CCHS with Hirschsprung Disease and the prevalent symptom of constipation, apparent even in the absence of Hirschsprung Disease.[30]

CHAPTER FOUR: PUBLIC AWARENESS OF CCHS WITH 1st ATS STATEMENT, INTRODUCTION OF ACRONYM ANSD, AND EARLY CANDIDATE GENE STUDIES (APPROXIMATELY 1996–2002)

This chapter brought CCHS from a hidden pediatric respiratory control disorder to the main stage for pediatric and adult medicine worldwide with the first American Thoracic Society (ATS) Statement on CCHS.[2] The aim of the ATS Statement was to improve general knowledge of CCHS, expedite the diagnosis, introduce management and referral options for state-of-the-art diagnosis and treatment, and recognize the important concepts about respiratory control and state-related cardiorespiratory and autonomic function that can be learned from the care of children with CCHS. The 1999 ATS Statement on CCHS estimated 160–180 cases of CCHS worldwide, with recognition that this would be an underestimate. In that Statement the acronym ANSD (for autonomic nervous system dysregulation) was introduced, as was the suggestion that CCHS would be the most severe manifestation of respiratory and autonomic dysregulation. Recognizing the wide ramifications of the ANS (Fig. 1), two seminal segregation analysis studies of large cohorts of children with CCHS and age-, gender-, and ethnicity-matched controls were undertaken. From these, CCHS grew from a rare control of breathing deficit to become the model for ANSD.[4,31] Specifically, symptoms of ANSD in CCHS probands were now apparent in multiple organ systems including but not restricted to respiratory, cardiac, sudomotor, ophthalmologic, neurologic, and enteric. Comparison of ANSD within these systems in children with CCHS as compared to control subjects, led to a more clear understanding of the ANS in health versus disease.

The expanded understanding of ANSD in CCHS and the relationship to Hirschsprung Disease led to study of candidate genes related to Hirschsprung disease. Twenty patients were reported with unique protein-altering mutations in receptor tyrosine kinase (*RET*);[32–36] glial cell derived neurotrophic factor (*GDNF*);[32] endothelin signaling pathway 3 (*EDN3*);[35,37] brain-derived neurotrophic factor (*BDNF*);[38] human aschaete-scute homologue gene (*HASH1*);[39,40] paired-like homeobox gene 2A (*PHOX2A*);[39] *GFRA1*;[39] bone morphogenic protein 2 (*BMP2*);[41] and endothelin converting enzyme 1 (*ECE1*).[41] Three other reports indicated an absence of *RET*[42] and *RNX* mutations.[43,44] Though these studies did not identify the disease-defining gene for CCHS, they focused attention on the network wherein the disease-defining gene for CCHS would eventually be discovered.

Hints toward the familiality of CCHS emerged by 2001. Familial recurrence data include one report each of affected monozygotic female twins,[45] sisters,[29] male-female sibs,[5,30] and male-female half sibs[46] with CCHS. Five women diagnosed with CCHS as children gave birth to one normal infant, two infants with CCHS, one infant with likely CCHS confounded by severe immaturity and bronchopulmonary dysplasia, and one infant with a delayed diagnosis of CCHS.[47,48] Report of a child with CCHS born to a woman who had neuroblastoma as an infant[49] added to the premise of a transmitted genetic component in the phenotypic spectrum of ANSD and CCHS. Further, ANSD was studied in a case–control family design,[4,31] which provided important confirmatory evidence for a genetic basis to CCHS, therefore regarded as the most severe manifestation of a generalized ANSD.[2,30]

CHAPTER FIVE: *PHOX2B* BASIC SCIENCE STUDIES (APPROXIMATELY 1997–2002)

This chapter would be read parallel to Chapter Four as this is the era of discovery of the Paired-Like Homeobox 2B (*PHOX2B*) gene. The *PHOX2B* gene, whose mRNA is composed of 3,074 nucleotides, encodes a protein of 314 amino acids characterized by a homeodomain sharing a significant homology with other paired-type homeobox gene family members. There are two alanine-rich repeats in the C terminus of the protein, the larger one being composed of 20 alanine residues. *PHOX2B* encodes a highly conserved homeodomain transcription factor known to play a key role in the development of ANS reflex circuits in mice,[50,51] whose expression is restricted to several classes of differentiating neurons in both the peripheral and central nervous system.[52] Consistently, *Phox2b−/−* mice show lack of intestinal innervation and of all central and peripheral neurons that express noradrenergic traits.[51]

CHAPTER SIX: *PHOX2B*: FROM IMPORTANT TO DISEASE-DEFINING, CLINICALLY AVAILABLE TESTING, AUTOSOMAL DOMINANT INHERITANCE PATTERN, AND MOSAICISM IN A SUBSET OF PARENTS (APPROXIMATELY 2003–2005)

In 2003, *PHOX2B* was found to be the disease-defining gene for CCHS.[41,53] *PHOX2B* contains a repeat sequence of 20 alanines in exon 3 which was reported to contain in-frame duplications of 15–27 nucleotides, leading to expansion of the repeat tract to 25–29 alanines on the affected allele in 18 of 29 (62%) French CCHS cases.[53] These expansions were reported as *de novo* since they were not present in eight sets of parents of the CCHS cases. Two of 29 (7%) CCHS cases had *PHOX2B* frameshift mutations. None of these *PHOX2B* mutations was present in controls. Concurrent to this study, Weese-Mayer et al.[41]

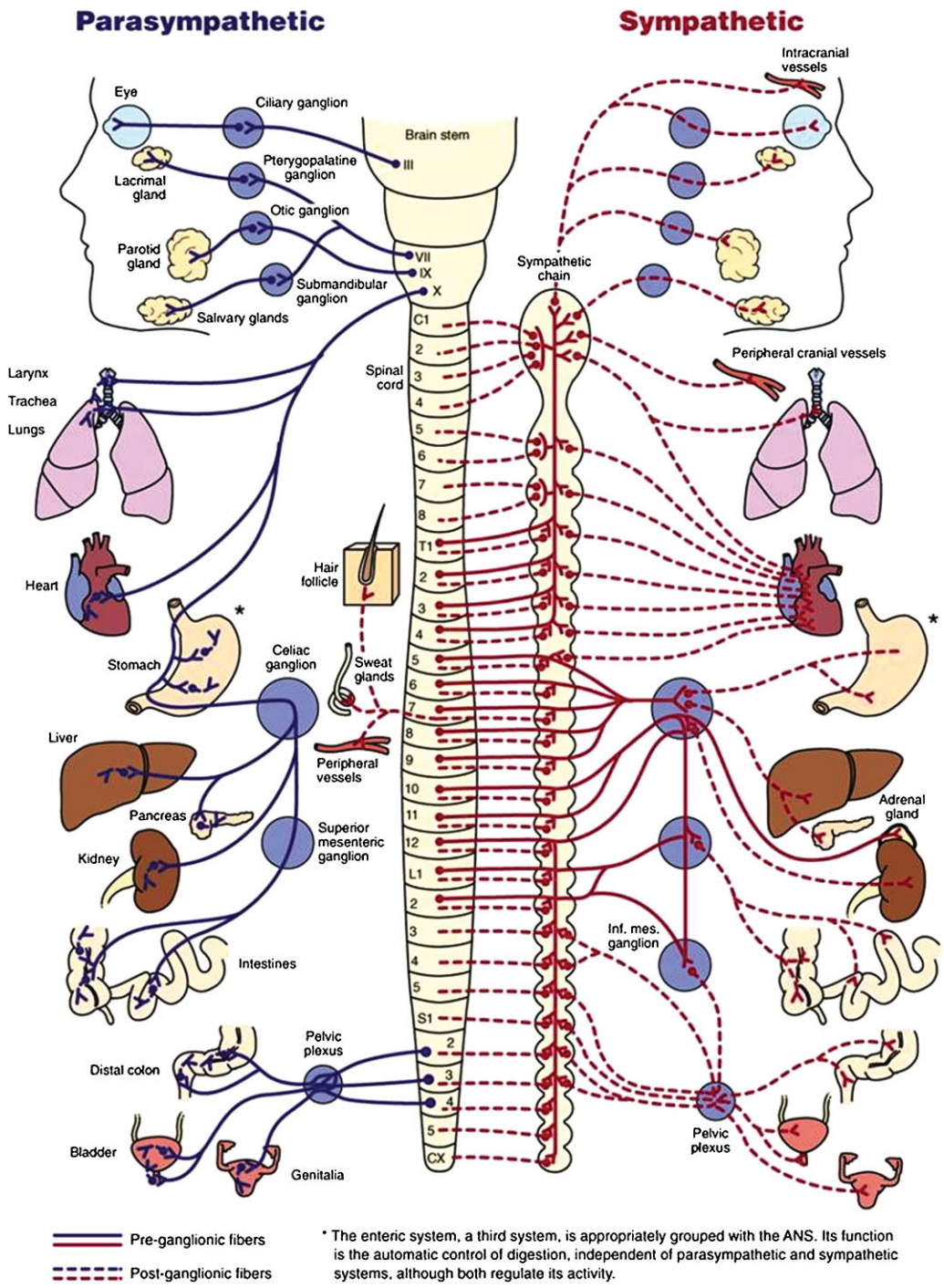

Fig. 1. Schematic of the ANS. This schematic figure demonstrates the organ systems affected by the sympathetic and parasympathetic nervous systems. The enteric system is not shown, but is grouped with the ANS.

focused on genes involved in the early embryology of the ANS, identifying *PHOX2B* exon 3 polyalanine repeat expansions of 25–33 repeats in 65 of 67 (97%) CCHS probands. Of the two remaining CCHS cases a nonsense mutation (premature stop codon) in *PHOX2B* was identified in one patient and the other was later found to have a polyalanine repeat expansion in *PHOX2B*, after a sample mix-up at the lab of origin was resolved.[54] Collectively, Weese-Mayer et al.[41] identified mutations in exon 3 of the *PHOX2B* gene in 100% of the 67 children with the CCHS phenotype, indicating that *PHOX2B* is *the* disease-defining gene in CCHS. None of the *PHOX2B* expansion mutations were present in 67 gender/ethnicity-matched controls. This study noted an association

between polyalanine expansion length and severity of autonomic dysfunction (number of ANSD symptoms).[41] Four of 97 parents of CCHS cases demonstrated mosaicism for the polyalanine expansion,[41] suggesting that not all CCHS-causing mutations occur *de novo*. In the three cases in which the child of a CCHS proband was affected, the mother passed the same expanded allele to the affected child. Likewise, mosaic parents passed the same expanded allele to the affected child, thus demonstrating autosomal dominant inheritance of CCHS and stable transmission of the *PHOX2B* mutation. Further, Weese-Mayer et al.[41] established a clinically available assay (*PHOX2B* Screening Test; Fig. 2) for the diagnosis of CCHS using a simple and accurate method for detecting and sizing the repeat sequence associated with the polyalanine tract expansion (patented; proceeds support CCHS research) which could be used for prenatal diagnosis, family testing and diagnosis of individuals with relevant symptoms.

Subsequent to the above studies, *PHOX2B* polyalanine repeat expansions were found in 4 (40%) and a *PHOX2B* insertion frameshift mutation in 1 (10%) of 10 CCHS cases in Japan.[39] Sasaki et al.[39] used the same methodology as the French,[53] likely under-detecting *PHOX2B* expansion cases as later determined. In 2004, Matera et al.[18] identified *PHOX2B* polyalanine expansion mutations of 25–33 repeats in 21 (88%) and heterozygous frameshift mutations in 2 (8%) of 24 CCHS cases from Italy, Germany, and The Netherlands. This study confirmed the correlation between the size of the *PHOX2B* expanded allele and the severity of both the respiratory phenotype and associated symptoms.[41] Matera et al. also demonstrated that in standard PCR reactions, the CCHS-associated expanded allele can remain undetected due to difficulty amplifying the GC rich polyalanine region of *PHOX2B*; thus, the *PHOX2B* mutation rate may be underestimated as a result of the amplification-induced allele drop out, as likely occurred in the original 2003 French and Japanese publications. Using assays designed to amplify GC-rich regions, Trang et al.[55] (re)analyzed the French patients and identified a *PHOX2B* mutation in 91% of 34 cases and Trochet et al.[19] found *PHOX2B* mutations in 93% of 174 subjects with CCHS from multiple nationalities, including 7 of the 9 "mutation-negative" patients reported by Amiel et al. in 2003.[53] Berry-Kravis et al.[54] and Weese-Mayer et al.[56] reported *PHOX2B* mutations in 184 subjects (in 2006) and collectively more than 350 subjects (in 2008) with CCHS, (apparent 100% sensitivity and specificity of detection), in a cohort primarily from the US, with 10% of patients from abroad. Thus far it seems unlikely that there will be other genes for the typical CCHS phenotype, most of which is explained by what is known about the *PHOX2B* gene. However, there is always a possibility that a few rare cases will be typical for CCHS and negative for a *PHOX2B* mutation, with ultimate explanation by some other gene.

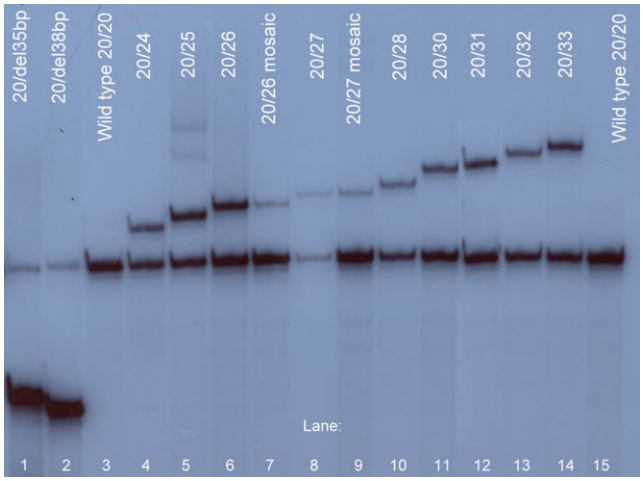

Fig. 2. Polyacrylamide gel electrophoresis *PHOX2B* Screening Test. This represents the methodology used for the *PHOX2B* Screening Test. Shown are *PHOX2B* polyalanine repeat expansion mutations (PARMs) by polyalanine repeat size and non-PARMs (NPARMs) for the most common CCHS-causing *PHOX2B* genotypes identified with the *PHOX2B* Screening Test compared to the wild type (Lanes 3 and 15) product. Lanes 1–2: Analysis of CCHS causing NPARMs 722del35 and 722del38. Lanes 4–14: Analysis of some of the known PARMs. Lanes 7 and 9 are mosaic carriers of CCHS-causing PARMs. Lane 7 is a mosaic parent of the proband identified in lane 6. Lane 9 is a mosaic parent of the proband identified in lane 8. Note that the band intensity of the expanded allele is much lighter than that of the wild-type allele in lanes 7 and 9. Also note that although the overall intensity of the signal in lane 8 is low, both the expanded and wild-type bands are similar in intensity indicating a full carrier of the expanded 27 repeat allele.

As recently summarized[3] (www.genereviews.org), the range for number of repeats in the polyalanine expansion on the affected allele in patients with CCHS is 24–33.[18–20,28,39,41,54,57] The polyalanine expansion mutation was not found in any of 482 controls from the above-cited publications, nor among 1,520 healthy individuals in a population study in Taiwan.[58] Six in-frame contraction variants with only 7, 13, 14, or 15 repeats in the polyalanine repeat tract, of unclear physiologic significance, have been reported in three CCHS cases[41,54,59] who harbor an additional polyalanine expansion or NPARM mutation, but are also found in ~3% of seemingly normal controls,[18,41,53,60,61] CCHS parents,[28,41,59] and a small subset of patients with vague symptoms suggestive of autonomic dysregulation and/or sporadic hypoventilation or apparent life threatening events, but not the constellation of symptoms characteristic of CCHS.[59]

PHOX2B Mutations in CCHS

A mutation in the *PHOX2B* gene is requisite to a diagnosis of CCHS (Fig. 3). More than 90% of subjects with CCHS will be heterozygous for an in-frame 12–39

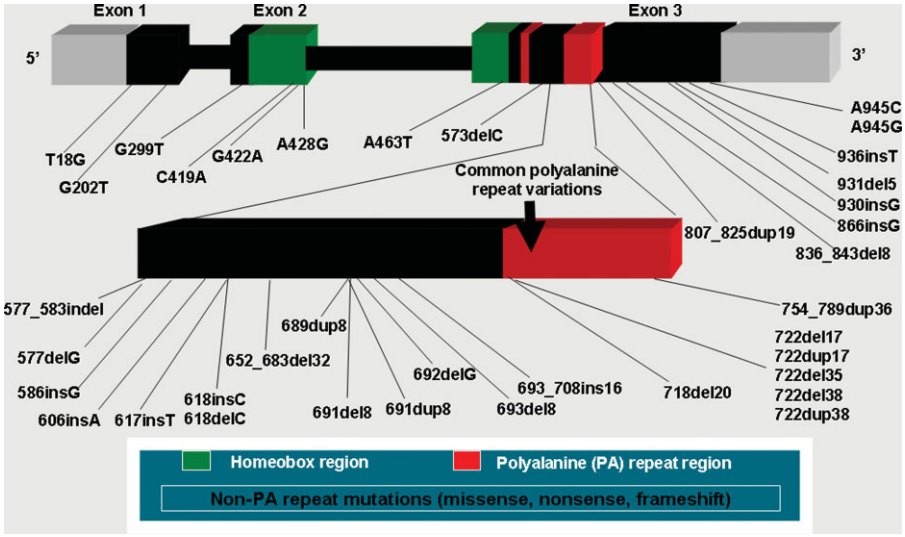

Fig. 3. Schematic for the *PHOX2B* gene with location of all CCHS-associated mutations described to date in *PHOX2B*. All polyalanine repeat mutations (PARMs) are located within the second polyalanine stretch of exon 3. Nearly all NPARMs identified thus far are found at the very 3′ end of exon 2 or in exon 3. These data represent all published literature as well as the current data from the authors.

base-pair polyalanine (PA) expansion repeat mutation (PARM)[3,59] coding for 24–33 alanines in the mutated protein and producing genotypes of 20/24 to 20/33 (the normal genotype would be referred to as 20/20). The remaining ~10% of patients with a CCHS phenotype will be heterozygous for a non-PA repeat mutation (NPARM)[3,59] (including missense, nonsense, and frameshift) in the *PHOX2B* gene. Genotypes 20/25, 20/26, and 20/27 are the most common PARMs, though an increasing number of more rare PARMs are being identified monthly (Fig. 4).

Non-polyalanine repeat mutations (NPARMs)[59] were reported in association with CCHS by groups in the US;[41,54,56,59,62] Italy,[18,28,63] Japan,[39] France,[19,53] Germany,[33,64,65] Australia,[66] and China.[67] At this time, 67 individuals with CCHS and NPARMs in *PHOX2B* have been described worldwide, and mutations include predominantly frameshift mutations (52/67, 78%), but also nonsense (2/67, 3%), missense (11/67, 16%), and missense with stop codon alteration (3/67, 3%) (Figs. 3 and 4).

Most NPARMs are *de novo* and with rare exception produce a severe phenotype with need for continuous ventilatory support, Hirschsprung disease with extensive enteric involvement, and an increased tumor risk.[19,54] Recurrent 35- and 38-base pair deletions, causing frameshift beginning in the first codon of the polyalanine repeat, produce the most severe phenotype, suggesting a specific mutational mechanism. A small minority of NPARMs are associated with a high incidence of Hirschsprung disease but milder disease in terms of the cardiorespiratory features of CCHS, and incomplete penetrance in at least three families.[54] The most prevalent predictor of a *PHOX2B* NPARM in a potential CCHS proband is Hirschsprung disease. A few similarly located frameshift mutations (618delC, 577delG) have been inherited and are variably penetrant in families,[18,54] suggesting that −1 frameshifts in this area may produce a milder disease phenotype than other frameshift mutations. The c.422G > A and c.428A > G mutations, leading to p.R141Q and p.Q143R respectively, have also each been found in several unrelated probands and, together with the c.299G > T (p.R100L) mutation,[62] are the only missense mutations yet identified in CCHS probands. In addition,

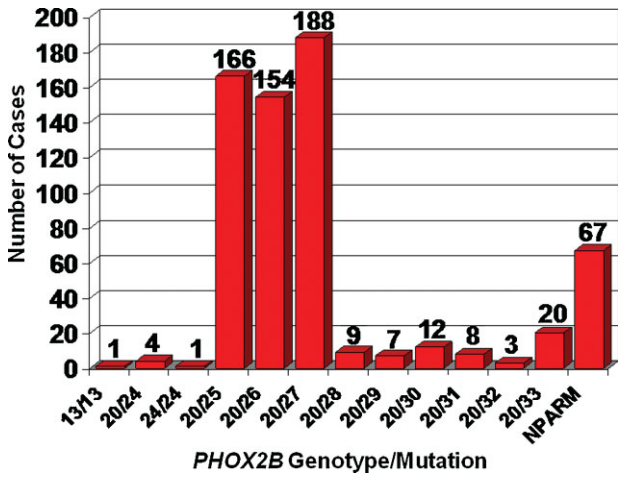

Fig. 4. Number of *PHOX2B* CCHS-related mutations internationally. These data represent all published literature as well as current data from the authors for the polyalanine repeat mutations (PARMs) and the non-polyalanine repeat mutations (NPARMs). The most common genotypes are 20/25, 20/26, and 20/27. Because the phenotype of the cases with 20/24 and 20/25 is more mild, it is anticipated that these cases are underrepresented at the present time.

the c.419C > A (p.A140E) has recently been reported in later presentation CCHS, both isolated and associated with HSCR.[28,57] The majority of CCHS-associated NPARMs are found at the end of exon 2 or in exon 3 (Fig. 3).

Mosaicism in Subset of Parents of CCHS Probands

Most expansion mutations occur *de novo* in CCHS probands, but in 5–10% of cases they are inherited from a seemingly unaffected parent. A distinction is needed between germline inheritance and somatic occurrence of the *PHOX2B* mutation among this subset of parents. Incomplete penetrance has been demonstrated when certain *PHOX2B* mutations are present in all cells (including reproductive cells of the germline) of individuals believed to be unaffected. These latter *PHOX2B* mutations, the 24 and 25 repeat polyalanine alleles and a few NPARMs, may produce somewhat milder, although variable, phenotypic effects in the CCHS-affected children or other family members.[18,20,41] Conversely, somatic mosaicism, due to post-zygotic mutations, has been observed among a subset of parents of typical CCHS probands carrying *PHOX2B* polyalanine alleles larger than 25 repeats and non-PA mutations.[19,28,41,54]

Somatic mosaicism for an expanded polyalanine *PHOX2B* allele was first reported by Weese-Mayer et al.[41] in four parents out of 54 available families (7.4%) with a CCHS proband. Trochet et al.[68] subsequently identified somatic mosaicism in 10 parents out of 124 available families (8.1%) of CCHS patients. Thus roughly 8% of probands will inherit the mutation from a mosaic parent. In both studies, mosaicism was detected in DNA extracted from parents' peripheral leukocytes by observing a lighter signal from the expanded allele than from the normal allele, in contrast to the pattern seen in subjects with CCHS.

A quantitative estimate of the somatic mosaicism in unaffected parents has recently been reported. DNA amplification products from asymptomatic carriers of alanine expansions ranging from 25 to 31 repeats were loaded on a DNA automated sequencer and the expanded and normal alleles were visualized as output peaks whose underlying area was directly proportional to their respective amounts. The mutant peak was expected to represent 50% of the *PHOX2B* alleles in the heterozygous individuals who inherited the mutation, but it was found to range from 9% to 35% in DNA from parental leukocytes; this percentage was confirmed in studies of fibroblast and saliva DNA from a subset of these mosaic parents.[57] In another study mosaic individuals were identified as "outliers," with less signal in the peak corresponding to the expanded allele.[28] While somatic mosaicism for PA expansions larger than 25 repeats was demonstrated in these studies, no asymptomatic carrier of a 25 repeat allele was found to be a mosaic; so in these cases lack of the CCHS phenotype can be ascribed to reduced penetrance of a germline mutation.[28,57] These data support the hypothesis that germline PA expansions larger than 25 repeats are fully penetrant and by extension, asymptomatic carriers may only be found in association with significant degrees of somatic mosaicism. Notably, the CCHS phenotype has not been associated with any degree of somatic mosaicism thus far, an observation which suggests a germline origin for expansion mutations in most, if not all, affected CCHS patients.

Autosomal Dominant Inheritance of CCHS and the *PHOX2B* Mutation

Detection of the same *PHOX2B* mutation in parent-child pairs and observation of somatic mosaicism in some unaffected parents for the mutation observed in their affected child clearly established an autosomal dominant inheritance pattern for CCHS.[41,68] Most parents of affected children with CCHS do not carry a mutation at all, indicating a high *de novo* mutation rate in affected individuals. The 24 and 25 repeat PARMs and some of the NPARMs may be found in the germline of asymptomatic parents of children with CCHS and even other family members, suggesting these mutations are inherited as dominant with incomplete penetrance.[18,20,22,23,54,57] Family members who carry such variably penetrant NPARMs and do not have CCHS may show other ANSD phenotypes, including Hirschsprung disease or neuroblastoma and those with 24 or 25 repeat PARMs and variably penetrant NPARMs may be presymptomatic, presenting in later childhood or adulthood.

Because of the heritability of the *PHOX2B* mutations, genetic counseling is essential for individuals diagnosed with CCHS, their parents and, in some cases, specific family members. There is a 50% chance of transmitting the mutation, and therefore the disease phenotype, to each child born to a CCHS proband. If an unaffected parent is mosaic for a *PHOX2B* mutation (parent identified because of an affected child), there will be up to a 50% chance of recurrence in any subsequent child. Mosaic individuals can be assumed to have a new mutation as the mutation cannot be inherited in mosaic fashion. Therefore, only children of these mosaic parents (not other family members) would be at risk to have the mutation. The *PHOX2B* Screening Test is more sensitive than the *PHOX2B* Sequencing Test for diagnosis of mosaicism, so for parental screening the Screening Test is recommended.[71] If unaffected parents carry a germline mutation (i.e., a 24 of 25 repeat PARM) there may be numerous other family members who carry the same mutation without overt symptoms. In this case, genetic testing is indicated for all persons in position in the pedigree to

inherit the mutation. In so doing the mutation can often be traced back until the individual in whom the mutation originated. To assess recurrence risk in families, ALL parents of a CCHS proband should have genetic testing done to rule out mosaicism (PARMs of 26 or more alanines and severe NPARMs) or a non-penetrant carrier state (24 and 25 PARMs and mild NPARMs). Prenatal testing is available and can be carried out for individuals with or without CCHS who are known germline mutation carriers or recognized as somatic mosaics. Despite negative testing of the parents of a proband with CCHS, germline mosaicism cannot be ruled out and prenatal testing for subsequent pregnancies should be considered. Prenatal testing allows parents optimal information with which to make an informed decision with a range of possibilities from elective abortion to a fully prepared delivery room.

CHAPTER SEVEN: IDENTIFICATION OF LATER ONSET PRESENTATION OF CCHS IN ADULTS, TODDLERS, AND CHILDREN AND UNDERSTANDING OF *PHOX2B* MECHANISMS IN CCHS (APPROXIMATELY 2004–2008)

Later Onset Presentation of *PHOX2B* Mutation-Confirmed CCHS

Though the term ''congenital'' as used in CCHS historically connoted presentation in the newborn period, patients presenting with later-onset CCHS (LO-CCHS) have been described.[18–24,26–28,57,72] With increased awareness of CCHS, discovery that *PHOX2B* is the disease-defining gene for CCHS, and recent availability of diagnostic tests for *PHOX2B* mutations, an increase in diagnosis of LO-CCHS with presentation in later infancy, childhood, and adulthood is anticipated.

LO-CCHS is *not* a separate entity from CCHS; it reflects the variable penetrance of the *PHOX2B* mutations with the fewest extra alanines (genotypes 20/24 and 20/25) or rarely an NPARM that at times may require an environmental cofactor to elicit the phenotype. Careful review of the medical history for individuals with observed hypoventilation subsequent to the newborn period often indicates ''missed'' signs/symptoms compatible with prior hypoventilation and other disorders of autonomic regulation from the newborn period. A heightened clinical suspicion of LO-CCHS, with prompt testing for a *PHOX2B* mutation, may avert potential life-threatening decompensation or neurocognitive impairment. Attention to report of delayed ''recovery'' from anesthesia, sedation, a severe respiratory illness, or treatment for obstructive sleep apnea should heighten suspicion of LO-CCHS. Unidentified environmental or genetic factors that might impact the variable penetrance of the 20/24 and 20/25 mutations as well as some missense mutations[73] need to be considered. Review of digital facial photographs (to evaluate for the characteristic CCHS facies), 72 hr Holter monitoring (to identify prolonged sinus pauses), any physiologic evaluations documenting ventilation both while awake and asleep (to identify alveolar hypoventilation), a hematocrit (for polycythemia), a bicarbonate level (for signs of compensated respiratory acidosis) and chest X-ray, echocardiogram, or electrocardiogram (signs of right chamber enlargement or pulmonary hypertension) should be completed. If severe constipation is reported, a barium enema or manometry should be performed to identify regions of potential aganglionosis. All patients with diagnosis after the neonatal period would be considered as LO-CCHS and can be distinguished from other syndromes of mild alveolar hypoventilation by the presence of a *PHOX2B* mutation.

Understanding the Relationship Between *PHOX2B* Basic Science Studies and CCHS

A novel genetically modified mouse was recently developed, bearing the frequent CCHS-causing expansion from 20 to 27 alanine residues in the *Phox2b* polyalanine tract.[74] The heterozygous *Phox2b*27Ala/+ offspring of the founder chimeras had irregular breathing, did not respond to hypercapnia, and died soon after birth from central apnea. Postmortem examination showed specific loss of Phox2b-expressing glutamatergic neurons in the retrotrapezoid nucleus/parafacial region, thus demonstrating the essential role of a specific population of medullary interneurons in driving proper autonomic breathing.[74]

Though polyalanine regions are present in several transcription factors and in-frame duplications in these sequences are emerging causes of human genetic diseases, particularly congenital development defects,[75,76] the normal function of polyalanine tracts and the effects of their expansion are not understood. Polyalanine tracts have been found in repression motifs of several homeodomain-containing proteins, and may act as flexible spacer elements between functional domains.[77] These expansions can result in protein intracellular aggregation, thereby affecting the physiological localization and the gene transactivating function of the wild-type protein.[19,63,78–81]

Knowledge about genes regulated by *PHOX2B* is limited. *PHOX2A*[82] and TLX2[83] encode transcription factors that control downstream processes involved in survival and differentiation of specific neural structures. *PHOX2B* directly regulates expression of tyrosine hydroxylase (*TH*) and dopamine beta hydroxylase (*DβH*), two genes that encode enzymes involved in catecholamine biosynthesis,[84,85] required for specification of noradrenergic peripheral and central neurons,

including those involved in sympathetic and parasympathetic systems. Most of the activity of the *PHOX2B* promoter depends on an auto-regulatory loop in which the transcription factor PHOX2B binds and transactivates its own promoter.[86] *PHOX2B* expression has an inverse correlation with *MSX1*, a homeobox transcription factor whose expression strongly up-regulates the Delta–Notch pathway genes. This pathway induces inhibition of proneural genes (leading to reduced neuronal differentiation), and suggests an interaction between the Delta-Notch differentiation regulatory pathway and the *PHOX2B* gene, potentially accounting for a role of these genes in neuroblastoma biology.[87]

Finally, protein–protein interaction assays showed that *Phox2b* interacts with a specific domain of the Trim11 protein. Expression of *Phox2b* and *Trim11* genes was detected in the sympathetic ganglia of mouse embryos while *in vitro* forced expression of *Trim11* further increased Phox2b-mediated DβH transactivation. These results suggest a potential role for *Trim11* in cooperating with *Phox2b* to produce the noradrenergic phenotype.[88]

An improved understanding of the physiological role of *PHOX2B* and the molecular mechanisms underlying CCHS pathogenesis will be gained with identification of the mechanisms whereby *PHOX2B* mutations induce cellular dysfunction. *In vitro* protocols have tested *PHOX2B* mutations for potential disruption of the normal function of the protein with respect to transactivation of different target promoters, DNA binding, aggregate formation, and sub-cellular localization. When mutant *PHOX2B* constructs containing poly-Ala mutations were co-transfected with the *DβH* and *PHOX2A* regulatory regions, cloned upstream of the reporter *Luciferase* gene, a strict inverse correlation between the trans-activating ability of *PHOX2B* constructs and the length of the polyalanine expanded tract was observed.[19,63] Yet, the same *PHOX2B* polyalanine constructs had only a weak effect on the *TLX-2* promoter.[83] Significant reduction of the trans-activating activity of *PHOX2B* constructs bearing common polyalanine contractions on the *DβH* promoter was observed in a similar assay,[60] though this observation could not be replicated in a different cell system.[19] This suggests the need for additional investigation before concluding that polyalanine contractions may result in some disruption of *PHOX2B* function.

Fluorescence microscopy of COS-7 cells expressing PHOX2B proteins fused to a green fluorescent molecule indicates that the wild-type PHOX2B protein is present almost exclusively in the nucleus. However, transfection of PHOX2B constructs with increasing polyalanine expansion lengths induce partial or complete cytoplasmic mislocalization of the protein; large aggregates are associated with the longest polyalanine tract expansions, in increasing proportions of cells.[63] This aggregate formation has also been confirmed in HeLa cells,[19] suggesting that impaired subcellular PHOX2B localization likely results from the aggregation-prone effect of the polyalanine expansion that is not specific to cell type. These findings are consistent with the premise that mislocalization of the mutant protein is a pathogenetic mechanism that results in impaired transcriptional activity in the event of PARMs. *PHOX2B* DNA binding is reduced for PHOX2B proteins containing expansions of 29 alanines and above, with the spontaneous formation of oligomers making them unavailable for DNA binding.[19] Finally, in addition to functional haploinsufficiency, PARMs have been demonstrated to exert a partial dominant negative effect, interfering with the usual function of the wild type protein because of its abnormal aggregation with the mutant protein.[19,63]

In order to determine the fate of cells expressing *PHOX2B* PARMs, *in vitro* experiments have demonstrated that activation of the heat shock response by the naturally occurring antibiotic geldanamycin is efficient in preventing formation and inducing clearance of PHOX2B preformed polyalanine aggregates and in rescuing the PHOX2B ability to transactivate the DβH promoter.[19,63] Elimination of PHOX2B mutant proteins by cellular mechanisms of proteasome and autophagy, known to be involved in the clearance of polyglutamine and polyalanine aggregates, has been demonstrated in other disorders.[89] On this basis, it can be postulated that present *in vitro* studies, aimed at understanding the pathogenetic mechanisms of PHOX2B mutant proteins, have potential clinical significance.

Mutant PHOX2B proteins carrying missense, nonsense, or frameshift NPARMs severely impair transcriptional activity for the DβH and TLX2 promoters correlating, in the case of frameshift mutations, with length of the disrupted C-terminal sequence.[19,63,83] *PHOX2B* frameshift mutations have induced a 10–30% increased activation of the *PHOX2A* regulatory region.[63] Similar to PARMs longer than 9 extra alanines, frameshift, and missense mutations have mainly shown a complete loss of DNA binding, despite correct localization in the nucleus.[19,63] A recent study has confirmed these findings and demonstrated that NPARM mutant *PHOX2B* constructs retain the ability to suppress cellular proliferation, without promoting differentiation, suggesting a mechanism which might promote development of neural crest tumors[62] and explain the association of frameshift and missense *PHOX2B* mutations with risk of neuroblastoma.

CHAPTER EIGHT: *PHOX2B* GENOTYPE/CCHS PHENOTYPE CORRELATION (APPROXIMATELY 2003, 2004, 2006–2008)

Despite identification that *PHOX2B* is the disease-defining gene for CCHS in 2003, authors continue to

publish research without confirmation that all "CCHS" subjects have *PHOX2B* mutations and without analyzing the data in a genotype/phenotype format. Owing to the crucial role of *PHOX2B* in the development of the ANS, relationships between *PHOX2B* genotype and the following aspects of the CCHS phenotype have been investigated.

Continuous Ventilatory Dependence

A relationship between the *PHOX2B* PARM genotype and the need for continuous ventilatory dependence has been demonstrated.[18,41] Continuous ventilation is rarely indicated in individuals with the 20/25 genotype. Variable awake needs, dependent upon level of activity, determine the ventilatory support among individuals with the 20/26 genotype. Individuals with genotypes from 20/27 to 20/33 very often require continuous ventilatory support especially as the expanded allele becomes larger. Adult-onset cases with the 20/24 or 20/25 genotype[20,22,23] have the mildest hypoventilation, primarily after exposure to respiratory depressants or severe respiratory infection, and are managed with nocturnal ventilatory support only. Most individuals with CCHS due to an NPARM require continuous ventilatory support[54] (Fig. 5), however there are families with NPARMs and neural crest tumors in which no individuals have obvious hypoventilation, and families with NPARMs and incomplete penetrance in which some individuals have CCHS and others with HSCR or no symptoms do not present with obvious hypoventilation.[69,70]

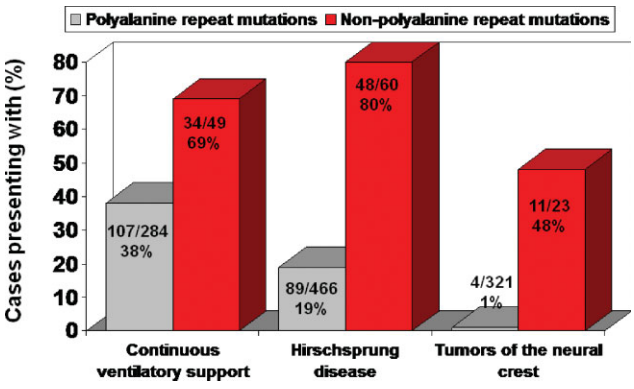

Fig. 5. Rate of continuous ventilatory support, Hirschsprung disease, and tumors of the neural crest in CCHS probands with polyalanine repeat expansion mutations (PARMs) in *PHOX2B* compared to CCHS probands with non-polyalanine repeat expansion mutations (NPARMs) in *PHOX2B*. CCHS probands included in this figure were compiled from all known cases reported in the literature including reports from groups in the USA, Italy, France, Japan, Germany, Australia, and China, where adequate clinical information was available. Neural crest tumor data derived from cases in which information was available and the proband had survived at least the first year of life. All polyalanine repeat probands with tumors had large (29–33 repeat) expansion mutations.

The results from reports of awake and asleep ventilatory and arousal responses,[90–92] mental concentration;[93] respiratory sensations,[11,12] physiologic response to exercise and leg motion,[9–12,94] and focal abnormalities on functional MRI[95–98] must be interpreted with caution as they likely reflect bias due to small sample size, reporting in the pre-*PHOX2B* era or without documentation of specific *PHOX2B* confirmation of CCHS, and inclusion of children who were able to sustain adequate ventilation during wakefulness at rest (so likely the majority of subjects had the 20/25 genotype based on the described phenotype). These results likely represent the physiology of only the mildest CCHS patients. Clarification in large cohorts, including a broad array of children and adults with genotypes of 20/24–20/33 as well as the NPARMs, will clarify the *PHOX2B* genotype/CCHS phenotype relationship in terms of respiratory control.

Hirschsprung Disease

Hirschsprung disease (HSCR) is more prevalent among cases of CCHS with NPARMs than those with PARMs. Specifically, HSCR is reported in 87–100% of NPARMs in contrast to 13–20% of PARMs (Fig. 5).[19,54] Although there are only a small number of cases, it appears that HSCR is not as frequent in families/individuals with NPARMs and no apparent hypoventilation. Among the PARMs, there are no reports of HSCR occurring in subjects with the 20/25 genotype. HSCR is rarely observed with the 20/26 genotype. Though a high occurrence of HSCR in individuals with the 20/27 genotype was reported in one cohort,[19] confirmation in a large cohort is needed to ascertain risk. Recent studies suggest that the *RET* gene may have a pivotal role as a modifier gene for the HSCR phenotype in patients with CCHS.[33,36,64,99]

Tumors of Neural Crest Origin

Tumors of neural crest origin are reported more frequently among individuals with NPARMs (50%) than among those with PARMs (1%)[19,54] (Fig. 5). However, among PARMs, only subjects with the 20/29–20/33[19,41,53,54] have been identified to have tumors of neural crest origin. These observations may allow the clinician to limit the number of children who require imaging in search of neural crest tumors.

Cardiac Asystoles

Gronli et al.[15] reported a correlation between the most common PARMs of 20/25 to 20/27 and length of R–R intervals on Holter monitoring. None of the children with

the 20/25 genotype had sinus pauses of 3 sec or longer. Yet 19% of individuals with the 20/26 genotype and 83% of individuals with the 20/27 genotype had pauses of 3 sec or longer. Cardiac pacemakers were implanted in 25% of subjects with the 20/26 genotype and 67% of subjects with the 20/27 genotype. Among the cases with the 20/26 and 20/27 genotypes who did not receive a cardiac pacemaker, two died suddenly and one has severe neurocognitive compromise. Notably, one individual with the 20/25 genotype, diagnosed in adulthood, has documented pauses of 8 sec and longer[23] as does another recently referred adult with the 20/25 genotype. These findings in *PHOX2B* genotype 20/25 adults diagnosed with LO-CCHS suggests that either (1) recurrent hypoxemia in undiagnosed mild CCHS will impact the cardiac autonomic phenotype or (2) children with the 20/25 genotype may not experience asystoles but with advancing age they, too, will demonstrate asystoles. These results emphasize the importance of annual 72 hr Holter recording in all cases of *PHOX2B* mutation-confirmed CCHS. The risk to individuals with the NPARMs remains unknown at this time.

Symptoms of ANSD

Weese-Mayer et al.[41] and Patwari et al.[100] demonstrated that an increased number of polyalanine repeats was associated with an increased number of symptoms of ANS dysregulation, but not of ANSD-affected systems involved. Though these measures of ANSD were ascertained from review of medical records, scripted questionnaires, and physiologic assessment, they did not include specific tests to assess autonomic function. As a preliminary step, these results suggest that parents and physicians should anticipate more symptoms of ANSD among children with the most extra alanines (i.e., genotypes 20/27–20/33).

Facial Dysmorphology

Todd et al.[101] described a characteristic facies in CCHS among children between the ages of 2 years and early adulthood. These faces were generally shorter and flatter, typically showing an inferior inflection of the lateral segment of vermilion border on the upper lip (lip trait). The characteristic box-shaped face observed in CCHS results from a face that is short relative to its width, though not overtly dysmorphic. Using five variables to characterize facies, 86% of the CCHS cases and 82% of the controls were correctly predicted. Genotype did not predict these defining variables, but the limited number of cases with higher numbers of repeats (only five cases with 30–33 repeats) may have precluded identification of a significant correlation between the number of polyalanine repeats and measures of the CCHS facial phenotype.

Dermatoglyphics

Todd et al.[102] studied dermatoglyphic pattern type frequency, left/right symmetry and genotype/phenotype correlation in CCHS, and determined that dermatoglyphic pattern type frequencies were altered in CCHS cases versus controls. Specifically, an increase of arches in females, and ulnar loops in males, was reported, with the largest differences for the left hand and for individuals with both CCHS and Hirschsprung disease. No significant association was found between the number of polyalanine repeats in the *PHOX2B* genotypic category and dermatoglyphic pattern frequencies in the CCHS study groups.

CHAPTER NINE: FROM SEMINAL CASE REPORTS TO MODEL FOR TRANSLATIONAL AND TRANSITIONAL AUTONOMIC MEDICINE (2009 AND BEYOND)

Between 1970 and the present time, clinicians and physician-scientists have dedicated themselves to understanding CCHS and determining optimal management. Consequently children with CCHS, living with the aid of technology, have often thrived while participating in normal childhood activities including attending school, graduating from high school and college, getting married, and finding steady employment. In contrast, children receiving suboptimal and inconsistent management were left with significant disabilities, and achievement of lesser accomplishments, remaining wholly dependent on their families.

Formation of Centers of Excellence for the evaluation and management of individuals with CCHS has allowed physician-scientists to study large cohorts of *PHOX2B* mutation-confirmed children with CCHS, resulting in a better understanding of the underlying nature of the disease and more full characterization of all aspects of the phenotype in relation to ANSD. We now understand that different mutations in the *PHOX2B* gene have different implications for the severity of an individual patient's disorder. Knowledge of the specific *PHOX2B* genotype/mutation guides clinicians to assess specific clinical abnormalities or risks, and knowledge of parental *PHOX2B* testing results allows for improved family planning. Some of these correlations have been described in this review, but many areas remain to be understood. By continuing to study individuals with CCHS in a consistent manner, with thorough examination of each organ system affected by the ANS and with collaboration from experts in each related organ system, we can more fully understand CCHS and improve the quality of life for affected children and adults. In the unfortunate event of death, it is hoped that families will agree to autopsy. Study of frozen (in contrast to fixed) brain, brainstem, carotid and aortic bodies, adrenal glands, autonomic plexus, sympathetic

chain, and the entire enteric tissue will allow for further delineation of biologic abnormalities in CCHS.

Taken together, CCHS is a strong example of translational and transitional medicine. It represents the success of collaboration between clinicians and basic scientists, and the success of collaboration between Pediatric and Adult Pulmonologists, Intensivists, and other specialists as children born with CCHS mature into fully functional adults because their phenotype could be anticipated and consistent management provided. In so doing, these children with a seemingly orphan disease,[103] are contributing to our understanding of the basic function of the ANS as it applies to health and disease. Likewise, children with CCHS can benefit from these discoveries and CCHS can serve as a keystone for investigating a growing number of other disorders within the rubric of ANSD,[104] particularly those with Respiratory and Autonomic Disorders of Infancy, Childhood, and Adulthood (RADICA).

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