The Sixth International RASopathies Symposium:

Precision Medicine: From Promise to Practice

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Abstract

The RASopathies are a group of genetic disorders that result from germline pathogenic variants affecting RAS-mitogen activated protein kinase (MAPK) pathway genes. RASopathies share RAS/MAPK pathway dysregulation and share phenotypic manifestations affecting numerous organ systems causing lifelong and at times life-limiting medical complications, RASopathies may benefit from precision medicine approaches. For this reason, the Sixth International RASopathies Symposium focused on exploring precision medicine. This meeting brought together basic science researchers, clinicians, clinician scientists, patient advocates and representatives from pharmaceutical companies and the National Institutes of Health. Novel RASopathy genes, variants and animal models were discussed in the context of medication trials and drug development. Attempts to

define and measure meaningful endpoints for treatment trials were discussed, as was drug availability to patients after trial completion.

Keywords

RAS, kinases, therapeutics, Neurofibromatosis, Noonan syndrome, CFC syndrome, Costello syndrome

Introduction

The promise of precision medicine is based on the premise that understanding the molecular abnormalities driving medical conditions will enable targeted drug treatment on a patient-specific level. The hope for precision medicine is that treatment tailored to an individual's disease-causing genomic, metabolic and pharmacogenomic variants will result in improved outcomes. The RASopathies, a group of conditions caused by germline pathogenic variants affecting the RAS/ERK mitogen activated protein kinase (RAS/MAPK) pathway, are well positioned for targeted treatment because they can have severe and life-threatening manifestations. These may be congenital, and/or occur throughout the patients' life. Candidate drugs exist throughout the RAS/ERK pathway, given the major role of this signaling pathway in driving cancer. The Sixth International RASopathies Symposium, held August 2-4, 2019 in Baltimore, Maryland, USA, aimed to further precision medicine approaches by highlighting the genetic causes of RASopathies, and their biological effects resulting in medical problems. Here, we share the proceedings of this meeting and invite interested parties to participate in this research effort.

Novel Pathogenic Variants and Novel Genes

RASopathy genes, including several RAS proteins (*HRAS*, *KRAS*, NRAS, *RRAS*, *RRAS2, RIT1*), RAS exchange factors (*SOS1, SOS2*), GTPase activating proteins (*NF1* and *RASAL1*), E3 ubiquitin ligases (*CBL*) and downstream kinases functioning as signal transducers in the MAPK cascade (*RAF* and *MAP2K* genes), have been extensively reviewed. Session 1, chaired by Marco Tartaglia, focused on additional genes recently implicated in RASopathies and newly characterized circuits controlling RAS-MAPK signaling. Phenotypic features associated with pathogenic variants in a subset of these genes were discussed. Finally, two talks were dedicated to the biology of SYNGAP1 and the disorder caused by SYNGAP1 haploinsufficiency.

Martin Zenker presented variant and clinical data from a large cohort of patients with Noonan syndrome collected in the NSEuroNet consortium. He focused on Noonan syndrome resulting from *LZTR1* variants (OMIM #616564 and #605275). The collected data suggest that, aside from *PTPN11*, *LZTR1* is among the most commonly mutated genes implicated in Noonan syndrome (similar in frequency to *RAF1* and *RIT1*). The phenotypes associated with dominant or recessive *LZTR1* variants fall within the Noonan syndrome clinical spectrum, and hypertrophic cardiomyopathy is common. Sixty five percent of those with *LZTR1* variants have dominant Noonan syndrome. In the remaining cases the mode of transmission is recessive or remains uncertain, as functional characterization of identified missense variants has not been performed, or because systematically scanning the noncoding portions of the gene may reveal additional variants. Notably, the distribution pattern of dominant and recessive LZTR1 variants is largely mutually exclusive, with the former, all missense, clustering within the solvent exposed portions of the N-terminal Kelch domains that mediate substrate binding, and the latter, including both missense and truncating variants, scattered throughout the entire coding region and adjacent intronic regions.

Anna Sablina reported that complete loss of Lztr1 function is embryonic lethal in mice, and Lztr1 haploinsufficiency recapitulates Noonan syndrome, with major features including reduced growth, craniofacial dysmorphia and cardiac defects, including cardiac hypertrophy. She discussed LZTR1 biology and evidence establishing its role in controlling RAS signaling. LZTR1 encodes a member of the BTB/POZ protein superfamily that functions as an adaptor for the cullin 3 (CUL3) ubiquitin ligase complex, a multi-subunit RING-class E3 ligase implicated in protein mono- and poly-ubiquitination. Mass spectrometry studies revealed that LZTR1 and CUL3 form a complex implicated in RAS ubiquitination. Nonetheless, the interaction studies indicate that multiple RAS subfamily members may be substrates of this complex. Notably, LZTR1-CUL3-mediated RAS ubiquitination does not appear to promote RAS degradation; instead, it alters RAS sub-cellular localization by impairing RAS association with membranes. New data indicate that disease-associated LZTR1 variants can affect RAS ubiquitination by disrupting LZTR1-CUL3 complex formation or diminishing the LZTR1-CUL3 complex interaction with RAS proteins, resulting in defective RAS ubiquitination and enhanced RAS signaling.

From abstracts submitted by junior investigators, three were selected for platform presentations. Pau Castel recently showed that the RIT1 protein is a target of LZTR1-mediated ubiquitination (Castel et al., 2019). He reported on an *Lztr1* knock out mouse model. Heterozygous mice were healthy and failed to show Noonan syndrome features. Homozygous mice were embryonic lethal due to extensive hemorrhage between embryonic days E15.5 and 18.5. *LZTR1* homozygous embryo hearts showed hypertrophic cardiac valves and sporadic septal defects. After back crossing to a different, more permissive background (129Sv), the homozygous knockout mice survived and showed a typical NS phenotype including small size, craniofacial features, cardiomegaly, and splenomegaly. Like children with biallelic *LZTR1* variants, the homozygous knockout mice had increased RIT1 levels. This mouse model can be used to study the pathogenesis of *LZTR1*-associated NS and to test pharmacological treatments.

Karen Gripp presented the clinical phenotype associated with de novo missense variants in *PPP1CB*, which encodes the protein phosphatase 1 catalytic subunit beta (PP1C). Major features include short stature, relative macrocephaly, distinctive facial features, congenital cardiac defects, pigmentary anomalies, and variable cognitive deficit, all common characteristics of Noonan syndrome and other RASopathies. The subjects carrying PPP1CB pathogenic variants have a phenotype resembling Mazzanti syndrome in which a recurrent activating variant in SHOC2 (p.Ser2Gly) causes a disease characterized by features resembling Noonan syndrome and distinctive ectodermal abnormalities, also called Noonan syndrome-like disorder (OMIM# 617506). Affected individuals have loose anagen hair (slow growing hair with a defective root structure that is easily pulled from the scalp; OMIM# 607721). Subjects with SHOC2 or PPP1CB variants show behavioral anomalies and cognitive defects, GH deficiency, and dark skin as additional common features (Gripp et al., 2016; Ma et al., 2016). No cancer has been reported in the 16 individuals with PPP1CB variants; the small number of cases does not yet allow genotype-phenotype correlations.

Pablo Rodriguez-Viciana presented the positive modulatory role of a PP1C, SHOC2, and MRAS complex in RAS-MAPK signaling. SHOC2 is a scaffold protein mediating RAS-promoted activation of the ERK MAPK cascade in response to extracellular stimuli. The SHOC2 protein consists almost entirely of leucine-rich repeats, and is a positive modulator of RAS-ERK signaling because it mediates membrane translocation of the catalytic subunit(s) of protein 1 (PP1C). PP1C is required for RAF1 activation and the PP1C/SHOC2/MRAS complex specifically promotes RAF1 dephosphorylation at a conserved inhibitory serine residue (Ser259), which in turn enables protein dimerization and activation. Data indicate that RASopathy-associated SHOC2, MRAS and PPP1CB mutants each cause enhanced MEK phosphorylation by promoting an augmented or more stable binding of the three proteins forming the MRAS/SHOC2/PPP1CB complex (Young et al., 2018). Of note, MRAS/SHOC2/PP1C complex-dependent and -independent mechanisms of RAF activation exist, which are thought to differentially modulate RAS-ERK signaling output in varying cellular contexts.

Frank McCormick analyzed the interaction between two RASopathy proteins, the *NF1* gene product, Neurofibromin, and SPRED1. SPRED1 is necessary for localization of Neurofibromin to active RAS. The EVH1 domain of SPRED1 binds to the GAP domain of NF1, allowing interaction of neurofibromin with RAS-GTP. Phosphorylation of serine 105 of SPRED1, within the EVH1 domain, appears key to this process. RASopathy disease-associated variants cluster at this interface and abolish NF1-SPRED1 binding. He described experiments showing that NF1 exists as a high-affinity homodimer, in head-to-tail conformation, with unidentified contact points. Full-length NF1 is relatively inactive as a GAP for RAS, compared to NF1 fragments. The dimerization of NF1 suggests the potential for dominant negative effects of certain disease-associated variants, which may result in divergent mechanistic, and hence phenotypic, effects. Missense substitutions of NF1 codons 844-848, associated with particularly severe disease manifestations (Koczkowska et al., 2018), are candidates for these effects. The half-life of NF1 mutated at codon 848 appears significantly reduced in vitro.

Two talks focused on SYNGAP1 and the neurodevelopmental disorder (OMIM# 612621; mental retardation, autosomal dominant 5) resulting from SYNGAP1 haploinsufficiency, with a particular focus on the clinical and functional overlap with RASopathies. Gavin Rumbaugh and Jimmy Holder outlined the clinical features of patients with pathogenic loss-of-function variants in SYNGAP1, which mainly include epilepsy, developmental delay, cognitive deficits, autism spectrum disorder and other behavioral abnormalities. SYNGAP1 encodes a protein that contains a RAS-GAP-related domain, which is highly expressed in brain and is characterized by alternative isoforms. This GAP largely localizes to dendritic spines in neocortical pyramidal neurons. It negatively controls RAS function and also regulates the activity of other small GTPases (e.g., RAP and RAB proteins). It is possible that the enhanced signal traffic through multiple GTPases of the RAS superfamily contribute to the clinical phenotype of patients with defective SYNGAP1 function. Therefore, further research assessing the significance of RAS signaling dysregulation associated with SYNGAP1 haploinsufficiency is required to define the SYNGAP1-related disorder as a RASopathy.

New Functions of RASopathy Genes

Emma Burkitt-Wright moderated a session starting with Shin-ichi Inoue presenting on the metabolic phenotyping of a novel *HRAS* G12S mouse model of Costello syndrome (CS) (OMIM# 218040) (Oba et al., 2017). Heterozygous animals have a phenotype reminiscent of human CS, with craniofacial features and cardiomyocyte hypertrophy, as seen in other mouse models of RASopathies (Araki et al., 2004; Schuhmacher et al., 2008; Urosevic et al., 2011). Less clearly related to human CS, but reminiscent of other *HRAS*-mutated mouse models (Schuhmacher et al, 2008), nephromegaly with fibrosis was identified. Mice fed a high fat diet were resistant to weight gain, but excess mortality was seen. Histological and biochemical investigation revealed microvesicular hepatosteatosis and impaired fatty acid oxidation: tandem mass spectrometry showed elevated blood acylcarnitines C16 and C18 (C18, C18.1 and C18.2) and lower fasting ketone levels than in wild-type animals. Decreased mitochondrial β-oxidation gene expression and lower blood glucose were demonstrated in mutant mice, after either 30-minute fast or intraperitoneal glucose injection. These findings may have relevance to the hypoglycemia observed in patients with CS.

Therapeutic Inhibitors: Clinical Trials and Preclinical Studies

Elegant work is ongoing to understand the dynamics of RAS-ERK signaling in RASopathies, and to test ways to block this pathway for therapeutic benefit, as envisioned in precision medicine. Targeting RAS itself, and/or up-stream and down-stream proteins in the RAS-MAPK cascade are being studied. Darryl McConnell discussed RAS as a potential drug target because RAS proteins play a central role in oncogenic signaling. Activated RAS promotes the formation of RAF homo- and hetero-dimers, which in turn induce downstream signaling. A known oncogene since 1982, one in eight tumors is driven by KRAS, making it a promising drug target. Unfortunately, there are no effective therapeutic agents against KRAS, because it lacks the easily targeted deep binding pockets present in kinases. Boehringer Ingelheim developed two KRAS exchange factor (SOS1) inhibitors, BI-2852 and BI-3406, which are effective at a nanomolar range. Boehringer Ingelheim maintains an open portal (*openme.com*) whereby access to these agents can be requested. At present, these agents are available for non-clinical studies only.

Neal Rosen's keynote lecture covered oncogenic variants in *RAF1*, and differentially activated feedback loops that develop after RAS-ERK activation. The different frequencies of pathogenic variants in the RAS-ERK pathway genes were evaluated in a set of sporadic cancers. BRAF variants classified into three groups. Of 200 mutated BRAF alleles, V600E is by far the most frequent, and acts through a Class I, classically activating, mechanism. Substitutions of codon 600 are also constitutively active, and like V600E result in mutant BRAF proteins that signal as monomers, while a substitution mutation involving the neighboring residue, E601G, is also activating, but signals as a homodimer. These Class I activating mutations result in profound feedback inhibition of RAS-GTP and are Ras-independent. In contrast, Class II mutated BRAF proteins are Ras-dependent, and bind tightly to Ras. Class II variants are 'kinase dead' mutations because they do not rescue the phenotype of H-, N-, and K-RAS deficient (Rasless) MEFs, and MEK inhibitors do not show efficacy against their effects. Class III BRAF variants require an additional driver mutation for a phenotypic effect, such as a loss of function variant in NF1, a combination observed in melanoma and lung cancer.

RAF proteins can homo- or heterodimerize. Early RAF inhibitors were monomer selective. Second generation RAF inhibitors inhibit ERK signaling by disrupting BRAF-containing dimers, including BRAF homodimers and BRAF-CRAF heterodimers, but not CRAF homodimers or ARAF-containing dimers. Such inhibitors appear effective in histiocytosis driven exquisitely by Ras-ERK signaling (Yao et al, 2019). Residual signaling through CRAF may permit a favorable side-effect profile of BRAF inhibitors. In contrast, MEK inhibitors, which block the pathway without leaving a collateral route for signal transduction, are associated with cutaneous and other side-effects. The MEK inhibitor trametinib has a very long half-life with low maximum tolerated doses, and infrequent dosing (such as once every 4 days) is one potential means to modulate pathway transduction while minimizing toxicities. Strategies taking into account the complex dynamics of these signaling networks will be necessary to explore effective therapies for the various conditions associated with aberrant Ras-ERK signaling.

PTPN11, the gene most commonly mutated in Noonan syndrome and Noonan syndrome with multiple lentigines (NSML), encodes Src homology region 2-containing protein tyrosine phosphatase 2 (SHP2), a non-receptor phospho-tyrosine phosphatase. Pre-clinical and clinical data demonstrate that SHP2 promotes tumor progression in leukemia and solid tumors. Ben Neel presented work indicating that SHP2 blockade can overcome resistance to MEK inhibitors in many cancers. The total range of substrates for activated Ras remains controversial, but the resistance of tumor cells to MEK inhibition appears intrinsic and hence unlikely to be overcome by combined inhibition of receptor tyrosine kinases (RTK) and MEK proteins. Compensatory reactivation of RAS is observed following MEK inhibition, and blocking SHP2 is a potential means to abolish this.

For example, NF1-mutated malignant peripheral nerve sheath tumors (MPNST) are frequently fatal, and single agent chemotherapy, even with pathway-focused MEK inhibitors, has not demonstrated dramatic effect, whilst dual blockade of RAS-ERK and PI3K/AKT pathways is precluded by excessive toxicity. RTK over-activation has been observed in MPNST cells treated with the MEK inhibitor trametinib, resulting in reactivation of RAS and MEK proteins and so preventing effective ERK inhibition. This reactivation is blocked by SHP099, an allosteric SHP2 inhibitor that prevents effects RAS-exchange, thereby decreasing RAS-GTP loading for wild-type RAS and "cycling RAS mutants", such as KRAS-G12C (Fedele et al., 2018). The observed effect of KRAS G12C inhibitors is potentiated by co-administration of SHP099 in RASless (H, N, and K-RAS null) mouse embryonic fibroblasts reconstituted with KRAS G12C and in G12C-expressing cancer cells. Furthermore, co-administration of MEK inhibitor with SHP099 potentiated tumor shrinkage in MPNST xenografts into sciatic nerve of nude mice. These results suggest promise for treatment of human MPNST with SHP2 inhibitors in combination with other agents blocking RAS-MAPK signal transduction.

Anton Bennett reported on the use of the multiple kinase inhibitor dasatinib in a *Ptpn11* mouse model of Noonan syndrome. A phosphoproteomic screen on Noonan syndrome and NSML mouse hearts with cardiomyopathy identified potential drug targets, including a hyper-phosphorylated protein, PZR, a transmembrane glycoprotein identified as an interacting partner and scaffolding protein for SHP2. NS and NSML associated mutations promote aberrant SHP2/PZR complexes driving promiscuous downstream signaling. Dasatinib, a multi-targeted inhibitor of *bcr-abl* and Src family kinases approved for pediatric cancers, inhibits PZR hyper phosphorylation in mice. Low doses of Dasatinib in a mouse model of NS improved cardiac function and in NSML prevented progression of hypertrophic cardiomyopathy.

In a second presentation selected from the submitted abstracts, Jae-Sung Yi reported on the use of a mouse model to demonstrate the interaction of protein zero-related (PZR) with SHP2 in the development of hypertrophic cardiomyopathy (HCM) in Noonan syndrome with multiple lentigines (NSML). Tyrosyl phosphorylation-defective PZR knock-in mutant mice were generated and intercrossed with NSML mice. Mice expressing tyrosyl phosphorylation-defective PZR alone exhibited normal heart development with normal heart and cardiomyocyte size. Those expressing tyrosyl phosphorylation-defective PZR and NSML were completely protected from the development of HCM and expressed Anf, Bnp, and Myh7:Myh6 levels comparable to controls. These results support targeting this adapter protein for the treatment of NSML cardiomyopathies.

In another presentation selected from the submitted abstracts, Maja Solman discussed the hematopoietic defects in a ptpn11a zebrafish knock-in D61G mutation generated by CRISPR/Cas9. The mutants survived to adulthood and displayed growth defects and alterations of craniofacial development. This analysis focused on hematopoiesis during early embryonic development. Mutant embryos had an expansion of hematopoietic stem cells and myeloid lineages including macrophages and neutrophils. Lymphocytes were unaffected. There was a decrease in the number of thrombocytes and erythrocytes. Attenuation of the stem cell expansion occurred using either a MEK inhibitor (Cl1040) or a PI3 Kinase inhibitor (LY294002), indicating that the hematopoietic defects were dependent on the MAP Kinase and PI3 Kinase pathway. Single cell RNA sequencing revealed an expansion of monocyte progenitors with an inflammatory transcriptional signature correctable with dexamethasone.

Alan Ho described a single-arm Phase II trial of the farnesyl transferase inhibitor tipifarnib in *HRAS* mutated squamous cell carcinoma (SCC). *HRAS* mutations occur in a molecular subset of Head and Neck SCC (HNSCC), converting *HRAS* to an active oncogene. Mutated HRAS is sensitive to tipifarnib, which ultimately prevents HRAS from binding to the membrane, thus rendering it inactive. This trial had two HRAS variant cohorts: Cohort 1: six patients with thyroid cancer; Cohort 2: 17 patients with HNSCC. The clinical endpoint was objective response rate. Of the HNSCC population, 6 patients had a partial response while 4 patients had stable disease. Tipifarnib may be effective in HRAS mutated recurrent or metastatic HNSCC (and to a lesser extent in thyroid cancer).

An overview of the NCI *Selumetinib in Pediatric Neurofibroma Study* was presented by Andrea Gross. Individuals with neurofibromatosis type 1 (NF1) are prone to develop benign plexiform neurofibromas (PNs), which result from the proliferation of nerve sheath Schwann cells, which form non-encapsulated tumors. About 10% of PNs transform into malignant peripheral nerve sheath tumors (MPNSTs), for which surgical resection is the only standard treatment. A study performed at the NCI Clinical Center using the MEK inhibitor selumetinib resulted in unprecedented shrinkage of PNs in NF1 (Dombi et al., 2016). These results prompted the Clinical Center to initiate the *Advancing Rasopathies Treatment* project, a multi-center approach to developing therapies for RASopathies and RAS driven pediatric tumors. A natural history study will be initiated and patients will be recruited to RASopathy clinics for interventional trials. The integration of natural history and treatment trials will substantially accelerate the understanding of RASopathies, and ultimately lead to the development of effective targeted therapies.

Rebecca Burdine described a system for screening the pathogenicity of possible RASopathy variants using CRISPR/Cas technology and zebrafish embryos (Jindal et al., 2017). In collaboration with Elizabeth Bhoj, MD, her group identified MAP4K4 as a novel RASopathy gene on patient exome sequencing. MAP4K4 appears to negatively regulate RAS signaling. Thus, loss of function and hypomorphic MAP4K4 alleles mimic the cardiac and craniofacial abnormities caused by increased RAS. This rapid model pipeline should facilitate verification of additional pathogenic RASopathy variants.

Maria Kontaridis discussed model systems of RASopathy cardiovascular defects. Using human induced pluripotent stem cell-derived myocytes, she could compare effects of variants in different RASopathy genes (Jaffré et al., 2019). Of note, in Raf1-associated NS mutant cells, while some cellular defects such as incomplete myocyte differentiation and fiber disarray could be rescued by blocking MEK or ERK1/2, cell hypertrophy was rescued by blocking the related kinase ERK5 (Li et al., 2019). These studies suggest drugs to be tested in preclinical models.

William Timmer presented a brief overview of several National Cancer Institute (NCI) resources: The NCTN Navigator contains tissue and blood samples from adult NCTN Phase III trials (80 trials, 50,000 patients, 600,000 specimens). The NCI Formulary maintains investigation agents. Finally, the NCI Cancer Therapy Evaluation Program maintains a portfolio of these investigation drugs available for study. Access to these resources requires a letter of intent that is peer-reviewed. Detailed information is listed on the NCI website.

Potential Endpoints for Clinical Trials in Rasopathies

Nancy Ratner moderated a session on treatment endpoints identified in model systems, which could be useful in human trials focusing on the brain and cardiac manifestations of RASopathies. Carlos Prada reviewed mouse models in which a HRas activating allele (HRasG12V) or Nf1 loss of function in mutant oligodendrocytes cause altered myelin structure in the brain, and motor dysfunction. In these mice, treatment with an antioxidant (N-acetyl-cysteine) ameliorated phenotypes (Mayes et al., 2013; Titus et al., 2017; López-

Juárez et al., 2017). Based on these results he opened a Phase 1 anti-oxidant clinical trial in children with NF1. He highlighted human studies showing that trans-magnetic stimulation (TMS) and the PANESS test each detect motor system deficits in children with NF1, and proposed use of these readouts, in addition to MRI endpoints, in the NF1 clinical trial.

Tamar Green discussed her ongoing study with in children with NF1 or Noonan syndrome (Green et al., 2017; Johnson et al., 2019). Preliminary data show that behavioral features such as attention problems and hyperactivity, as well as atypical behavior, appear more common in Noonan syndrome than in NF1. Both groups of children showed frequent attention problems and hyperactivity. Autistic features appear more common in Noonan syndrome than in NF1, and both groups of children showed frequent hyperactivity. Weaknesses in learning and spelling were observed. Measurements of brain region size in Noonan syndrome reveal reduced size of the basal ganglia, which correlated with hyperactivity. Diffusion tensor imaging measurements in Noonan syndrome showed reduced fractional anisotropy indicating possible loss of or aberrant myelin, similar to findings from NF1.

Susan Blaser reviewed neuroimaging findings in Rasopathies. She emphasized vascular defects including aneurysms and moya moya as late features of NF1, and more subtle changes in other Rasopathies (Cizmeci et al., 2018). Prenatal use of fetal three-dimensional ultrasound reconstructions can detect some RASopathy features at 22-25 weeks gestation. Structural changes in cortical folding and the corpus callosum, or cerebral white matter lesions may be detectable. Infratentorial changes including increased cerebral fluid or persistent venous sinuses, or a small posterior fossa with hypertrophy of its bony edges, may be present. An increase in Chiari 1 malformations has been observed.

Prenatal Findings, Manifestations, Diagnosis and Management

Pilar Magoulas moderated the session on prenatal manifestations, screening and diagnostic tools for RASopathies. Angie Jelin reviewed prenatal screening for RASopathies by gestational age. In the first trimester (11-14 weeks gestation), measurement of the nuchal translucency (NT) is possible. In the second trimester (18-22 weeks), an anatomy ultrasound can detect major malformations, whereas in the third trimester (>24 weeks gestation), ultrasound is particularly beneficial for monitoring growth and amniotic fluid levels. In a retrospective review of prenatal characteristics identified in fetuses later diagnosed with cardio-facio-cutaneous (CFC) syndrome, 10% had an abnormal 1st trimester ultrasound (3/29), 61% had an abnormal 2nd trimester ultrasound (25/41), and

79% had an abnormal 3rd trimester ultrasound (22/28). The most common first trimester findings included increased NT, cystic hygroma, and abnormal ductus venosus on doppler study. In the second trimester, common abnormal ultrasound findings included polyhydramnios, macrosomia with proportionate short long bones, macrocephaly, skeletal abnormalities, ventriculomegaly, abnormal lymphatic system (thickened nuchal fold, cystic hygroma, hydrops, ascites), renal abnormalities and cardiac defects (hypertrophic cardiomyopathy, pulmonary valve stenosis, septal defects, supraventricular arrhythmia). A 3-D ultrasound can show facial features that might otherwise be missed, such as hypertelorism, down-slanting palpebral fissures, long and marked philtrum, low-set posteriorly angulated ears, prefrontal edema, and thick lips. Comparing amongst RASopathies, fetuses with NS were more likely to show increased NT, distended jugular lymphatic sacs, cystic hygroma, hydrops fetalis, pleural effusion, polyhydramnios, cardiac defects, and renal anomalies. They were less likely to have fetal overgrowth and macrocephaly. Features more common in CFC syndrome include polyhydramnios, renal abnormalities, fetal overgrowth, pleural effusion and cardiac defects. Fetuses with CS were more likely to show polyhydramnios, fetal overgrowth, and macrocephaly. Prenatal NF1 is rarely diagnosed, however, case reports include findings of polyhydramnios, tumor growth, and macrosomia. Adverse perinatal outcomes including miscarriage, fetal demise and premature delivery occur at a higher frequency in fetuses with RASopathies.

Sandra Darilek presented prenatal screening and diagnostic testing options for RASopathies. Prenatal suspicion of a RASopathy can be confirmed after CVS or amniocentesis, either by testing for a known familial variant or through RASopathy panel testing. In a study of 845 prenatal cases that had RASopathy panel testing consisting of 9 genes, 74 variants were found in 72 cases (Leach et al. 2019). Of those, 31 variants were considered pathogenic/likely pathogenic, a large majority in *PTPN11* (81%). There were 43 variants of uncertain significance (VUS). The overall diagnostic yield was 3.7% (31/845) (Leach et al., 2019). Of the fetuses with positive results, 64.5% had a cystic hygroma only, and 20% had an increased NT only. The highest diagnostic yield based on fetal indication was cystic hygroma with 12% (20/162) having a pathogenic/likely pathogenic variant. Unpublished data from a single private practice collected over a 4-year period on 14 cases sent for RASopathies panel testing showed a diagnostic yield of 28.6% (4/14).

Prenatal screening options for RASopathies include two clinically available tests on cell free fetal DNA (cffDNA): PreSeek/Vistara available through Baylor Genetics/Natera and Resura available through Progenity. Preseek is a single gene cffDNA screening option that assesses 30 genes associated with various conditions and includes screening for 13 RASopathy genes. PreSeek/Vistara screening can be used for singleton pregnancies at >9 weeks' gestation, pregnancies with ultrasound finding suggestive of a disorder on the panel, or pregnancies where the father has a confirmed molecular diagnosis of a disorder on the panel. It cannot be used when the mother is affected. In a review of ~1600 samples tested using PreSeek, 475 had an indication of abnormal ultrasound or family history. There were 18 cases with positive result for a pathogenic/likely variant in a RASopathy gene. Half were *de novo* in the fetus, in 22% (4/18), the variant was identified in the mother and the fetus, and in 28% (5/18), the fetus was positive for a known familial variant present in the father. Of the *de novo* cases, variants were identified in *BRAF, HRAS, PTPN11* and *RIT1.* Indications for screening in positive RASopathy cases included increased NT, cystic hygroma, hydrops, shortened long bones, and father affected with NS.

Resura offers custom cffDNA screen for families with known inherited disorders. This is performed after 10 weeks' gestation and can take 4-8 weeks for test set-up and results. The assay is custom designed for the familial variant. Limited data is available on RASopathy specific cases, however, validation data reported that 57 cases were performed for benign variants and fetal genotype was confirmed via Sanger sequencing. Nine cases were performed for known pathogenic variants for autosomal recessive conditions. All fetuses were reportedly unaffected based on Resura and neonatal confirmation testing.

In summary, prenatal testing is available for RASopathies through CVS or amniocentesis. Prenatal screening through cffDNA testing is also available. However, this is not a diagnostic test so would require confirmatory prenatal or postnatal testing. The most common RASopathy genes diagnosed in the prenatal setting include *PTPN11*, *RAF1*, *KRAS*, *BRAF*, and *MAP2K1*. Genetic counseling is beneficial in assisting families in deciding which option(s) might be best for them and to provide anticipatory guidance if a prenatal diagnosis of a RASopathy is confirmed.

Clinical Trial Endpoints Roundtable

The session on clinical trial endpoints was moderated by Richard Klein. Since 1962, under Kefauver-Harris Amendments, the federal drug administration (FDA) requires effectiveness data derived from adequate and well-controlled clinical investigations. Endpoints should demonstrate patient benefit and be accurately measurable using validated instruments, reproducible over time, and across observers. Clinical endpoints demonstrate a direct effect on how patients feel, function or survive. Clinical outcomes may be represented by increased survival, improvement of cognitive symptoms, or improved quality of life.

Alternatively, surrogate endpoints are indirect measurements, such as radiographic images showing reduced tumor size, or blood chemistries. While these are not clinical endpoints in themselves, they may predict a positive clinical outcome. Surrogate endpoints can be used to support accelerated approval, which requires continued study once a medical product is approved demonstrating clinical benefit. Endpoints should also demonstrate cost effectiveness to ensure commercial success and availability through third party payers.

Karin Walsh discussed her study of MEK inhibition on neurocognitive function in children and adults with NF1. She described selecting sensitive and feasible outcome measures for this ancillary cognitive study. Performance based tests of reaction time, attention, and working memory were administered via a focused computer based cognitive battery (Cogstate). Real-world executive functions were assessed via parent-rated symptom questionnaire (Behavior Rating Inventory of Executive Function). There was a trend towards improvement in performance-based measures over the first 12 months of treatment, and significant improvement on parent-rated executive functions observable at the six-month follow-up and increasing by 12-months on therapy. Analyzing the possible impact of changes in pain and physical functioning is important in fully interpreting these results and additional research is necessary given the lack of a comparison group.

Pamela Wolters talked about the use of Patient Reported Outcomes (PROs) in descriptive and natural history studies as well as clinical trials. PROs (reported by the patients themselves) differ from clinician-reported outcomes (collected by a clinical investigator, physician or other health provider) and observer-reported outcomes (collected by caretakers) (Acquadro et al., 2003). General quality of life (QOL) scales evaluate a wide range of domains, including physical, social, and emotional functioning. Disease-specific QOL scales focus on specific functions affected by a specific medical condition, and symptom specific tools assess one symptom, such as pain, in any medical condition (Luckett et al., 2010). PRO measures may provide data on positive outcomes, like improvements in functioning, as well as negative effects, such as treatment-related toxicities. One main challenge in the use of PRO measures in clinical trials for RASopathies is that less self-reporting is achievable with young children and individuals with cognitive disabilities. Current approaches that can improve self-reported data collection for children with cognitive impairments include use of pictures, simplified language, more white space around text, and having information/questions read aloud to patients (Kramer and Schwartz 2017). The use of wearables may be a strategy to elucidate otherwise difficult to assess data (Slade et al., 2018). For example, increased physical activity might be used as a measure of reduced pain.

No validated PRO measures specific to RASopathies exist. Researchers need to evaluate PRO measurement scales to determine which are appropriate for RASopathy trials and to prioritize tools necessary to fill in data gaps. Benefits of getting PROs approved through the FDA Clinical Outcome Assessment Qualification Program may include faster review time, more meaningful outcomes, and quicker availability of new therapeutic approaches (U.S. Food and Drug Administration, 2009). However, the FDA's program for development and validation of PROs is a long, rigorous process.

Annie Kennedy from Parent Project Muscular Dystrophy (PPMD) talked about the organization working with industry, families, patients, and Certified Duchenne Care Centers to centralize data in the Duchenne Outcomes Research Interchange. Different registries have collected valuable, but siloed data. Even basic questions like how walking is measured can vary among researchers, leading to inconsistent and problematic results. PPMD brings data sources together and validates the data. She discussed the Duchenne Regulatory Science Consortium, formed under the Critical Path Institute to develop tools and optimize trial designs intended to accelerate the development of meaningful therapies. While tighter inclusion criteria can lead to faster results and earlier approval, payers may restrict access based on results in a limited study population. PPMD and other advocacy organizations were originally focused on regulatory approval, rather than data necessary to inform payers, which can result in restricted access. While patients, industry and regulators all prioritized biomarkers as important, payers did not. Payers are recognizing caregiver burdens and the effects on entire families with higher costs. Thus, quality of life measures might provide evidence of overall lower costs of care.

PPMD is leading efforts to evaluate the prioritization of outcomes through a broad range of stakeholders including patients, parents, methodologists, industry, physicians, clinicians and payers.

Advocacy Groups Panel on Clinical Trial Endpoints

A discussion was moderated by Michelle Ellis, an adult advocate with NS. The panel consisted of a representative from each advocacy group: Tuesdi Dyer from CFC International; Angel Thomas from Costello Syndrome Family Network; Alwyn Dias from Children's Tumor Foundation; Gregg Erickson from Neurofibromatosis Network; and Amanda Brown from NSF. Patient advocates highlighted pain, plexiform neurofibromas, intractable seizures, neurocognitive function and social skills as the most urgent treatment targets. Further studies on how RASopathies impact the lives of adults are requested. The heterogeneity of RASopathies, not only on the genetic level but also amongst individuals affected by the same syndromic condition makes identification of meaningful endpoints challenging. The audience was reminded that affected individuals are keen to participate in research studies. Adults deciding about such study participation were willing to accept a higher risk for themselves, in contrast to what they would accept for their child. Difficulties can arise when a treatment trial ends and the study drug is no longer available to participants.

Million Dollar Bike Ride 2018 and 2019 Grantee Reports

Kartik Venkatachalam described the use of Meclizine to attenuate hyperactive RAS-MAP Kinase signaling. RAS isoforms are specific to and dependent upon specific cholesterol-enriched domains for proper plasma membrane localization. Perturbations disrupting RAS localization attenuate MAP Kinase signaling. The recycling of these cholesterol moieties is dependent upon lysosomal exocytosis. Inhibition of lysosomal trafficking reduces plasma membrane cholesterol and inhibits MAP Kinase signaling in cells with activated HRAS. The anti-emetic, Meclizine, inhibits phosphate cytidylyltransferase 2 (PCYT2) which results in an increase in sphingomyelin sequestering and thereby lowers cholesterol. PCYT2 is also required for maintenance of mitochondrial iron homeostasis, preventing the translation of sphingolipid biosynthesis enzymes, thus regulating the gain of MAP Kinase signaling. Meclizine attenuates MAP Kinase signaling and thus may have potential use in treatment of hyperactive RAS-MAP Kinase based RASopathies.

Bruce Gelb reported on a Drosophila RASopathy model to screen a large 14,400 compound library of chemicals for potential drug development. Using the Drosophila RAF^{S257L} (a lethal model, 4% survive to pupae), each chemical was introduced at a variety of concentrations to find rescue from lethality. Four compounds (M1-M4) showed consistent rescue of RAF^{S257L} as well as reduced RAS/ERK activity *in vivo*. M1 was selected as the lead compound to advance due to its superior chemical structure. Thirty chemical analogs of M1 were developed and the flies treated again; several of these analogs showed improved efficacy. These analogs were further modified and several compounds further increased efficacy. Using a RAF1 induced pluripotent stem cell cardiomyocyte model, one compound normalized cardiomyocyte size. Future work includes testing the most promising compounds in 14 Drosophila RASopathy models with examination of eye and wing phenotype. RAS pathway signaling will be evaluated by western blot analysis to identify compounds with the potential to address multiple RASopathy subtypes.

In conclusion, significant therapeutic effects of pathway specific medications on life altering complications of RASopathies have already been reported, such as the use of selumetinib for plexiform neurofibromata in NF1 (Dombi et al., 2016) or, most recently, trametinib for hypertrophic cardiomyopathy in NS (Andelfinger et al., 2019). Ongoing research on drug development and delivery as discussed during this meeting promises further progress towards precision medicine for RASopathies and associated medical complications. Patients, clinician scientists, laboratory scientists, drug companies and regulatory bodies are coming together to promote such outcomes driven research.

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