CONFERENCE REPORT

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Recent Developments in Neurofibromatoses and RASopathies: Management, Diagnosis and Current and Future Therapeutic Avenues

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Neurofibromatosis type 1 (NF1) was the first RASopathy and is now one of many RASopathies that are caused by germline mutations in genes that encode components of the Ras/mitogen-activated protein kinase (MAPK) pathway. Their common underlying pathogenetic etiology causes significant overlap in phenotypic features which includes craniofacial dysmorphology, cardiac, cutaneous, musculoskeletal, GI and ocular abnormalities, and a predisposition to cancer. The proceedings from the symposium "Recent Developments in Neurofibromatoses (NF) and RASopathies: Management, Diagnosis and Current and Future Therapeutic Avenues" chronicle this timely and topical clinical translational research symposium. The overarching goal was to bring together clinicians, basic scientists, physician-scientists, advocate leaders, trainees, students and individuals

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with Ras pathway syndromes to discuss the most state-of-the-art basic science and clinical issues in an effort to spark collaborations directed towards the best practices and therapies for individuals with RASopathies.

Key words: capillary malformation–AV malformation syndrome; cardio-facio-cutaneous syndrome; Costello syndrome; neurofibromatosis; Noonan syndrome; Legius syndrome; Ras/MAPK; signal transduction pathway; RASopathy; therapy

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INTRODUCTION

The international symposium "Recent Developments in Neurofibromatoses (NF) and RASopathies: Management, Diagnosis and Current and Future Therapeutic Avenues" was held on September 30th and October 1st, 2013 in Cardiff, Wales, UK at the Radisson Blu Hotel. Clinicians, basic scientists, physician-scientists, clinical and molecular geneticists, advocate leaders, genetic counselors, trainees, students, and individuals with Ras/MAPK syndromes and their families attended. Nearly 150 registrants participated. This conference was organized under the auspices of Wales Gene Park, Cardiff. Educational funds were provided by NeuroFoundation, NF Ireland, Children's Tumor Foundation, International Costello Syndrome Support Group, Noonan Syndrome Association, NewGene, BioMarin, Heart Research Fund for Wales, and Sheila and Clive Owen. Professor Meena Upadhyaya chaired the symposium and welcomed all by sharing the history of neurofibromatosis type 1 (NF1) research in Cardiff, Wales, UK. Research started in 1983 with Dr. Susan Huson, under Professor Peter Harper's leadership, and now 30 years later, is still an active area of research. Dr. Upadhyaya became involved in NF1 in 1986 just as Dr. Huson was leaving Cardiff to take a position in London. The team was able to contribute toward the mapping of the NF1 locus a year later and subsequently made substantial contributions towards cloning of the gene, establishing protocols for molecular diagnosis including both constitutional and somatic mutation detection of NF1, understanding functional analysis of mutations and establishing genotype-phenotype correlations. Recently, the research team has focused on NF1-associated cancers in order to generate potential therapeutic targets. Dr. Upadhyaya explained that human genomics is revolutionizing medical genetics which has evolved from dealing with single gene-based syndromes to pathway-based groups of conditions. Accordingly, RASopathies constitute a group of syndromes that are caused by molecular lesions in the Ras/Mitogen-activated Protein Kinase (MAPK) pathway. It is estimated that over 50,000 people in the UK could be affected by RASopathies, so as genetic conditions, they are collectively not so rare. NF1 was, of course, the first RASopathy to be recognized, even if that wasn't obvious at the time; however, during the last few years many more RASopathies have been identified [Upadhyaya and Cooper, 2012]. In summary, this two day symposium achieved its goal by providing an open forum for attendees to share and discuss basic science and clinical issues setting forth a solid framework for future research, translational applications directed towards therapy and best practices for individuals with NF and other RASopathies. These proceedings provide the clinical and scientific communities with an executive summary of the clinical translational research symposium and to publish the abstracts from the platform presentations.

SUMMARY OF PRESENTATIONS

Session I was chaired by Eric Legius and Vincent Riccardi. This session reviewed the different forms of neurofibromatoses, the differential diagnoses and the molecular bases of the syndromes NF1, neurofibromatosis type 2 (NF2) and schwannomatosis. Complex NF1 is defined as unusual NF1 phenotypes and rare complications or manifestations of disease that are potentially life threatening or cause significant morbidity. Tumor burden and whole body MRI in NF1 was discussed in the context of plexiform neurofibromas (PNF) which can be seen in up to 50% of individuals with NF1.

Session II, led by Susan Huson and Meena Upadhyaya, focused on mouse modeling and preclinical testing in NF1. Discussion in this session focused on neoplasia in NF1. The first presentation described in NF1 there are two central issues in the field of solid tumors: (1) What the identity of the tumor source cells is? and (2) Whether solid tumors propagate in a clonal versus hierarchical model. The second presentation focused on translational research with genetically-engineered animal models for NF1 and the treatment of neurofibromas with MEK inhibitors.

Session III was moderated by Nancy Ratner and D Gareth Evans and centered on learning issues in NF1 and clinical trials for NF1. As discussed in this session, one of the major barriers to the successful implementation of clinical trials, of which many have focused on learning issues, is an incomplete appreciation of the heterogeneity inherent to this disorder.

Session IV was chaired by Rosalie E Ferner and David Gutmann and focused on NF2, schwannomatosis and related disorders. In addition, preclinical modeling and clinical trials were discussed for NF2. Merlin, the protein encoded by NF2, is a tumor suppressor which is haploinsufficient in NF2 and is lost in a variety of tumors. New drug targets and how they translate into early clinical trials were discussed.

Session V was led by Luis F Parada and David Viskochil. This session discussed the complexities of understanding the function of neurofibromin (Nfn), as well as the examination of social skill levels in NF1 individuals and the overlap of their social skills and autism. As was introduced in Session I, bone health is of great concern in individuals with NF1.

In Session VI, moderated by Bronwyn Kerr and Martin Zenker, the RASopathies were introduced. The RASopathies include NF1, Noonan syndrome (NS), NS with multiple lentigines, Costello syndrome (CS), cardio-facio-cutaneous syndrome (CFC), capillary malformation–arteriovenous malformation syndrome and Legius syndrome. Detailed clinical overviews were provided on CS and Legius syndrome.

Session VI was chaired by Katherine Rauen and Michael Patton. This session continued the detailed clinical overviews of RASopathies and included in-depth discussion of capillary malformation–ateriovenous malformation syndrome, CFC, NS and NS with multiple lentigines (formally known as LEOPARD). In addition, the session provided an over view of animal modeling in the RASopathies, as well as exploring treatment for these genetic syndromes.

SPEAKERS' ABSTRACTS

Diagnosing the Different Forms of Neurofibromatosis: A Practical Approach

Susan Huson. The different forms of NF are distinguished clinically by what combination of skin pigmentary changes, eye features and peripheral/central nervous system tumors the patient has. Neurofibromas are seen in NF1 and schwannomas in NF2 and schwannomatosis although there is some overlap including tumors with a mixture of histologies. Diagnosis is usually straightforward after clinical evaluation, although expert histological review of tumors and radiological review of imaging may be necessary. In childhood, the commonest presentation is of a child with multiple café-au-lait (CAL) spots referred as possible NF1. There are few other conditions in which a child will have multiple CAL spots without other obvious clues to a non-NF1 diagnosis. They occur in various ring chromosome syndromes but the child will usually have more developmental problems than seen in NF1 and other dysmorphic features. If the pigmentation varies in intensity and has very irregular edges, or follows large segments of the body then other diagnoses such as DNA repair syndromes, pigmentary miscegeny (the result of parents having very different skin coloring) or McCune-Albright need to be considered. If the CALs are limited to one or more body segments mosaic/segmental NF1 is likely. In children with less than six CAL the possibility of NF2 needs to be considered. Children with sporadic severe NF2 may present in childhood with peripheral nerve/skin schwannomas or characteristic eye changes causing visual problems. Recently recognized conditions like Legius syndrome and constitutional mismatch repair deficiency syndrome (CMMRD) mean the NIH diagnostic criteria for NF1 are no longer robust. Patients with Legius syndrome present with CAL spots and skinfold freckling indistinguishable from NF1, the distinguishing features clinically are the absence of Lisch nodules and neurofibromas. CMMRD is caused when a child inherits a mismatch repair gene mutation from each parent. The heterozygote parents may therefore have a family history that suggests hereditary non-polyposis colon cancer, although this is not always the cases particularly with the less penetrant *PMS2* gene. The skin phenotype of CMMRD consists usually of irregular hyper-pigmented and hypo-pigmented lesions. However, rare cases can have segmental or full blown NF1 with the somatic NF1 mutation occurring as a secondary event. These children are at an increased risk of childhood CNS and hematological malignancies and survivors develop colon cancer at a young age, inheritance is recessive. The NIH criteria include a first degree relative with NF1 by the above criteria. CMMRD and the fact that pure gonadal mosaicism (with unaffected parents having two children with NF1) is extremely rare means that siblings alone should not count in the diagnosis.

Complex NF1

Rosalie Ferner. The majority of individuals with NF1 manage well with the support of local clinicians, community services and family. However, specialized care is required for people with rare complications or unusual NF1 phenotypes and for disease manifestations that are potentially life threatening or cause significant morbidity. The Complex NF1 multi-disciplinary services were established in London and Manchester in 2009 with the aim or providing long-term education, management and support for people with complex disease and their families. The experience of the London unit was discussed and some retrospective and prospective clinical findings were highlighted. Currently 360 patients attend the service and 411 complex disease manifestations were identified in this cohort between 2009 and 2013. The commonest problem identified was symptomatic PNFs (99 patients) and the presentation was with one or more of persistent pain, neurological deficit or sphincter disturbance, or change in texture or size of visible lesions. Sixty patients have been followed-up or diagnosed with malignant peripheral nerve sheath tumor (MPNST) since 2009. Thirty-seven of 60 (61.6%) patients have survived at least five years from the initial diagnosis. In 34 individuals with pseudarthrosis, the tibia was the site most frequently involved and 24 patients required surgery including excision of dysplastic bone, external fixation with bone grafting and bone morphogenetic protein and below knee amputation in four cases. Fifty patients were diagnosed with brain glioma including pilocytic astrocytoma, fibrillary astrocytoma and two people with glioblastoma multiforme. Although many tumors remained indolent, twenty five individuals with gliomas required surgery and two had intracranial shunts for hydrocephalus. The commonest mode of diagnosis for 65 NF1 associated gliomas was neuroimaging for unrelated symptoms and nine children were diagnosed on visual screening. Thirty-four people required treatment for progressive visual impairment and five were treated after the age of 6 years. Erroneous diagnoses of NF1 included NF2, schwannomatosis and Cowden syndrome. Complex NF1 services will facilitate prospective data collection and the formulation of management protocols and outcome measures to promote high quality patient care.

Tumor Burden and Whole Body MRI in NF1

Said Farschtschi. NF1 is an autosomal dominant inherited tumor predisposition disorder with an estimated incidence of about 1:3000 and characterized by the occurrence of PNF in about 50% of patients based on whole body MRI studies. PNF are rarely identifiable by physical examination and are a major cause for morbidity in NF1 patients. Malignant transformation from PNF into MPNST is the most important cause of early death in the NF1 population. We are evaluating new therapeutic approaches in order to identify patients at risk. Whole body MRI might be used as a potential risk stratifier for NF1 risk groups. Recent studies were focusing on following questions:

1. Whole body tumor burden in children and adolescents with NF1
2. Growth dynamics of PNFs
3. Relation between cutaneous/subcutaneous and internal tumor burden
4. Internal tumor burden and risk of MPNST
5. Growth behavior after surgery of PNF

About 57% of 65 NF1 patients show internal PNF with 46% exhibiting tumor specific deficits such as pain and/or neurological impairments. PNF tend to cause complications in older children and adolescents more frequently than in young children. Growth rate of tumors is correlated with total internal tumor volume and inversely correlated with age. Patients without tumors are unlikely to develop new internal PNF. In terms of risk stratification subcutaneous but not cutaneous tumors can be used to estimate whole body internal tumor volume. Scalp subcutaneous neurofibromas are easy to detect and to count on cranial MRI and seem to be a valuable prognostic marker for internal tumor load. Additionally we found that a high number of PNF and/or a large tumor burden in young age are important risk factors for MPNSTs. After surgery regrowth of PNF was observed with a significant accentuation in young patients. After complete resection, regrowth of PNF seems to be very unlikely. Internal tumors are accurately identifiable by whole body MRI and volumetry of these lesions allows quantitative analysis of tumor progression. Longitudinal studies are necessary to assess the potential value of whole body MRI and volumetric analysis in clinical care, for example risk assessment for morbidity or malignant degeneration. Volumetric analysis of tumor burden in NF1 patients can be used to detect small changes over time. For best results it is recommended to use the same imaging parameters in all follow-up studies, such as field of view, imaging matrix, image resolution, echo time, repetition time and inversion time.

New Developments in Mouse Modeling of NF1

Luis Parada. Two central questions in the field of solid tumors are: The identity of the tumor source cells; and moreover, whether solid tumor propagates in a clonal versus hierarchical model. Answers to these questions are of importance because of the therapeutic implications. If the cell of origin for MPNST is in fact a rather unique and rare cell, then its isolation and study would be critical to better understanding the process and mechanism of tumor development. Likewise, if the cells propagate as an essentially equivalent mass of cells, then gross tumor shrinkage is an accurate assessment of therapeutic effectiveness. If however, the tumors develop in a hierarchical manner in which the cells with tumor propagation properties (cancer stem cells) represent only a subset of tumor cells, then therapeutic value can only be measured by the effects on the cancer stem cell population and not on the tumor as a volume as a whole. For this the putative cancer stem cell would have to be identified, isolated and analyzed. We have developed and studies mouse models of NF1 based MPNST to attempt to evaluate these processes and by extension to seek new therapeutic avenues to these intractable tumors. Our studies point to a relatively rare population of neural crest derived progenitors as the source of murine MPNSTs. Using this key piece of information, we have been able to more precisely analyze the molecular changes of the cell of origin as it becomes transformed into a malignant cell. These studies have led to the identification of the G-coupled receptor, CXCR4 as a critical component for the MPNST cells to proliferate. We have identified the intracellular pathways downstream of CXCR4, associated this pathway to human patient MPNSTs and devised novel strategies to therapeutically tackle these tumors. Using a variety of transgenic tools devised in the laboratory, we have further addressed the question of the cancer stem cell. Our preliminary studies with MPNST, based on much more advanced work on glioblastoma, give us indication that MPNSTs indeed grow in hierarchical manner. These preliminary findings are under rigorous validation. Together with the above mentioned studies, we have optimism that we will have successfully exploited our mouse models to learn more about MPNST development and to identify novel therapeutic opportunities.

Preclinical Testing in NF1

Nancy Ratner. NF1 patients are predisposed to develop benign PNFs that cause substantial morbidity. Surgery, the only standard treatment, is not feasible for most tumors. The NF1 gene product accelerates Ras-GTP hydrolysis to Ras-GDP and thus functions as a potent negative regulator of Ras. The Ratner laboratory developed a genetically engineered mouse model of neurofibroma, DhhCre; Nf1fl/fl, for use in preclinical testing; response is monitored by MRI imaging and volumetric analysis in conjunction with pharmacokinetic and pharmacodynamic readouts. The most effective neurofibroma therapy in this neurofibroma mouse model to date is drug candidates targeting inhibition of MEK1/2. New studies of the allosteric MEK inhibitor PD-0325901 using the DhhCre;Nf1fl/fl model were described. Results indicated that even lower doses than those previously tested (1.5 mg/kg/day) retain efficacy, and that no rebound effect occurred after dosing, although tumors did regrow. Also, early MEK inhibition, for 3 months, delayed but did not prevent neurofibroma growth. Recently published studies aimed at modeling white matter in NF1 and Costello syndromes were also described. Ratner's group induced Nf1 loss or HRas hyperactivation in mouse oligodendrocytes. Enlarged brain white matter tracts correlated with myelin decompaction, and, surprisingly, non-cell-

autonomous defects in perivascular astrocytes and the blood-brain barrier. Treating mice with the NOS inhibitor NG-nitro-L-arginine methyl ester or the antioxidant N-acetyl cysteine corrected cellular phenotypes. CNP-HRasG12V mice also displayed locomotor hyperactivity, which were rescued by antioxidant treatment. It will be interesting to determine if antioxidants can improve some behavioral deficits in RASopathy patients.

Learning Problems in NF1

Eric Legius and Thijs van der Vaart. NF1 is an autosomal dominant disorder characterized by multiple café-au-lait spots, benign tumors of the peripheral nerves and learning and behavioral problems. The NF1 gene codes for Nfn, a protein responsible for the down-regulation of activated RAS proteins. RAS signaling is highly increased in many malignancies. More recently it has been shown that the RAS-pathway is also very important for learning and memory. There are now a number of inherited conditions known that are caused by mutations in genes coding for key components of the RAS-pathway such as NF1, NS, LEOPARD syndrome, CFC syndrome, CS, and Legius syndrome. These disorders are all associated to some degree with learning and behavioral problems. More recently it has been shown that children with NF1 have a higher frequency of social cognition problems. Previous work by the group of Alcino Silva showed that hyperactivation of the MAPK pathway in inhibitory GABA-ergic interneurons in heterozygous *Nf1* knockout mice is responsible for a reduced synaptic plasticity leading to learning problems in these animals and that reversing of the hyperactive RAS pathway was able to acutely correct the learning problems in adult mice. One of the drugs successfully used in mice was lovastatin. This opened the door to clinical trials in humans with NF1. At this moment several trials using statins in children have been initiated and recently a larger trial by the Rotterdam and Leuven groups following up on the initial pilot trial was finished and did not show any benefit on cognition from taking simvastatin during a 12-month period.

Clinical Trials for NF1

David Gutmann. NF1 is a common neurogenetic condition characterized in part by defects in Ras regulation. While the discovery that the NF1 protein, Nfn, is a critical negative Ras regulator has opened the door to rational targeted drug therapies for NF1, one of the major barriers to the successful implementation of clinical trials is an incomplete appreciation of the heterogeneity inherent to this disorder. Numerous factors that contribute to clinical heterogeneity and limit our ability to deliver effective treatments for the medical problems arising in people with NF1 were discussed. Using NF1-associated brain tumors (optic gliomas), genomic/genetic factors have been found to influence glioma formation and clinical behavior . In addition, leveraging novel *Nf1* genetically engineered mouse strains, a series of experiments were described establishing the critical importance of the timing of somatic *Nf1* gene inactivation, the cell of origin, and the local cellular microenvironment to gliomagenesis and continued tumor growth. Strategies are needed to better dissect clinical heterogeneity as a critical step towards the design and execution of precision medicine for individuals with NF1. These strategies include (1) the development and utilization of enabling resources, such as the NF1 Patient Registry Initiative, clinically annotated DNA and induced pluripotent stem cell repositories (http://nfcenter.wustl.edu), and research-oriented patient clinical databases, (2) robust collections of informative preclinical small-animal models, and (3) an efficient clinical trials infrastructure. Over the past decade, the generation of accurate preclinical genetically-engineered mouse strains coupled with the establishment of the Department of Defense NF Clinical Trials Consortium has created a logical pipeline for drug discovery, early evaluation and translation to human subjects. The future stratification of individuals with NF1 into clinically distinct subgroups will provide exciting opportunities for risk assessment and targeted individualized therapies for this patient population.

NF2 and Related Syndromes

D Gareth Evans. NF2 is an autosomal dominant tumor predisposing condition caused by mutations in the *NF2* gene on chromosome 22. Individuals who inherit a mutant copy inevitably develop schwannomas on the vestibular nerves at some time in their lives. Most individuals develop bilateral disease in addition to schwannomas on other cranial and spinal nerve roots. Meningiomas affect about 70% of individuals at some time in their lives and low grade ependymomas are also common especially in the cervical spine. Management of NF2 requires a multidisciplinary team approach involving: neurosurgeons, otolaryngologists, neurologists, neuroradiologists and geneticists. NF2 has a birth incidence of 1 in 25–33,000 and a prevalence in the UK of 1 in 56,000. There are at least two other related syndromes to NF2 that cause predisposition to either schwannomas and/or meningiomas. Schwannomatosis is rarer than NF2 and causes painful predominantly spinal and peripheral nerve schwannomas with sparing of the cranial nerves. About 50% of inherited cases are due to germ-line mutations in the *SMARCB1* gene and about 10% of sporadic cases. There are continuing searches to identify remaining schwannomatosis genes by exome sequencing. Recently we identified the gene *SMARCE1* by this approach as the cause of a predisposition to spinal clear cell meningiomas and have now identified six families with germ-line mutations.

Preclinical and Clinical Trials for NF2

Oliver Hanemann. The validity of the human in vitro model of human schwannoma as model for merlin deficient tumors has been established. Our model reflects in vivo findings and emphasizes that schwannomas are genetically well defined and homogenous. Tyrosine kinase receptors are found to be overexpressed and activated in human schwannoma. These are mainly ErbB2, ErbB3, PDGFR, and Axl. Activation of these leads to downstream activation of the Ras/Mek/Erk and Pi3K pathway. The in vitro model allows one to test drug candidates. PDGFR inhibitors Sorafenib, Nilotinib, and Imatinib led to reduction in Erk pathway activity and proliferation. Sorafenib was most effective. Erb/EGFr Lapatinib showed less effect than Sorafenib and Nilotinib with the MEK inhibitor Selumtinib in between. The combination of Nilotinib and Selumtinib demonstrated to be quiet effective in vitro. Building on this, the status of

current clinical trials in NF2 was overviewed. The published data on avastin and lapatinib was summarized and the on-going Phase 2 trials with everolimus, the Phase 1 trial AR42 (HDAC inhibitor) and the Phase 0 trial with Sorafenib were discussed.

Understanding Neurofibromin Function—A Clinicians Perspective

Vincent M. Riccardi. The task of understanding Nfn, the gene product of the *NF1* gene, is substantially different from understanding the disorder, NF1, although the hereditary nature of NF1 pointed to the presence of a unique gene ultimately accounting for the disorder. The actual understanding of Nfn was initiated with the cloning of the *NF1* gene, more than a century after the disorder's definitive characterization. While modest progress has resulted from detailing and cataloging *NF1* mutations and deletions, and carrying out genotype–phenotype correlations among thousands of patients with NF1, these data merely establish a partial phenotype for *mutations* and *deletions*. They do not directly address Nfn function determined by the *wildtype* alleles. For example, innumerable investigators have labeled *NF1* a "*tumor suppressor gene*," keying off one element of the mutations' phenotypes. More accurately, we only know that, for a large number of *NF1* gene changes, there are many "*tumor contributor mutations*." Moreover, it is not even clear whether this attribution applies, on the one hand, to the array of sarcomas and other malignancies associated with NF1 or also, on the other hand, to the neurofibromas that are hallmarks of the disorder. Designating a neurofibroma to be a "tumor" is as much poetry as it is science. Over the long run, the neurofibroma will best be understood as a scar, a type of "wound-healing gone awry." Ribeiro and colleagues quite convincingly document this consideration, although they perpetuate the notion that the lesion, the neurofibroma, is necessarily a tumor and that diploinsufficiency for *Nf1* abrogates a "tumor suppression" function of *NF1*/*Nf1* wild-type alleles. Applying a clinical approach to the NF1 disorder, acknowledging that compromised attentiveness is the most consistent cognitive element of the disorder, one can characterize the *NF1* gene as an "*attentiveness suppressor gene*" and appropriately rename Nfn as *Attentin*. If the clinical approach has any merit at all, it is most likely to be fruitful in this regard: The wild-type *NF1* alleles contribute to human performance, with specific regard to functions of the central, peripheral and autonomic nervous systems, particularly when time is of the essence, when response "on the instant" is required for organism and species survival. This conclusion is reinforced by acknowledging the NF1 person's lean habitus and absence of diabetes, the attributes of *Saccharomyces cerevisiae* homologues of *NF1* and, finally, the most well-studied interactome element of *NF1* and *NF1* mutations, namely, "hyperactive Ras" or RasGTP "overdrive." This presentation had three elements: (1) Background material emphasizing previous work on NF1, the disorder, *NF1* mutations, and details of RasGTP "overdrive," as well as recent publications establishing a role for "*metabolic signatures*" and cell–cell "*cooperativity*" as cogent alternatives to *mutations* and *clonality* in understanding lesions characterized by cell accumulations, such as tumors and granulation tissue; (2) A review of relevant literature regarding the roles of aerobic glycolysis and substrate level phosphorylation under both physiological and pathological conditions, with particular regard to biochemical stress and physical confinement (tight places, such as myelin sheaths, filopodia), combined with a synthesis emphasizing the role of glycolytic metabolic signatures in considering therapeutic approaches to treating incipient and established neurofibromas; (3) A literature review documenting that mast cell sequestration of skin keratinocyte-synthesized vitamin D_3 (cholecalciferol) can account for both the low levels of calcidiol and/or calcitriol and thereby at least some NF1 skeletal and extra-skeletal abnormalities, including neurofibroma development and the potential for NF1 treatment with inhibitors of mast cell degranulation, for example, ketotifen.

Social Skills and Overlaps with Autism in NF1 Patients

David Viskochil. The prevalence of autism spectrum disorder (ASD) is purported to be increased in NF1, although this diagnosis is often inferred from parent-reported screening tools such as the Social Responsiveness Scale (SRS) rather than through direct evaluations using gold standard diagnostic assessments such as the Autism Diagnostic Observation Schedule (ADOS) or the Autism Diagnostic Interview (ADI). It is not known if the psychometric properties of ASD screening tools are appropriate for detection of possible autism in the NF1 population. We compared the rate at which children with NF1 screen positive for two ASD-specific screening tools [Modified Checklist for Autism in Toddlers (M-CHAT) and Childhood Autism Spectrum Test (CAST)] to the screen-positive rate of children from the general population. Medical records of children between the ages of 16 months and 11 years seen in the University of Utah NF Clinic were reviewed for an ASD screening questionnaire. Individuals scheduled for NF1 evaluation or follow-up assessment in the NF Clinic between September 2010 and June 2012 were included in this assessment. Of 342, 153 met criteria for NF1 or greater than 5 cafe-au-lait spots and 42 were between 16 and 48 months of age, while 111 were between 4 and 11 years of age. Of this group, 21 families completed the M-CHAT survey (mean age 30.5 months, range 19–47months, 12 males); and 46 completed the CAST survey (mean age 6.8 years, range 4–11 years, 25 males).

There were no statistically significant differences in the screen-positive rate for ASD in NF1 when compared to published control screen-positive rates, but mean CAST scores were higher in NF1 suggesting that school aged children with NF1 may exhibit more of the traits of ASD without reaching the threshold of screening positive for ASD. In reviewing the literature on prevalence of ASD in NF1, there is a tendency to rely on screening tools to infer an association of ASD symptoms without establishing a diagnosis of ASD. Although ASD characteristics are frequent in NF1, other issues such as cognitive deficits, learning problems, and attention deficit hyperactivity disorder are further pronounced and could influence the social adaptation phenotype. In her PhD dissertation at the University of Utah (1993), C. Dilts identified 3 key findings in her conclusion. One conclusion is "Motor skill deficits and their influences on adaptive behavior were the most frequent problem in

the NF sample across both cognitive and behavioral variables." A second observation is, "Examination of specific ratings on a Social Problems measure indicated that the phenomenon of being teased by others was the most commonly rated problem followed by the NF child acting too young." Finally, a third conclusion was that "Examination of the items on the Child Behavior Checklist Social Problems scale suggested that peer acceptance may interact when the child or adolescent with NF acts different (plays with younger children, clumsy) from age-level peers; that is, social problems may relate to maturational factors." It will be important for future studies to dissect the social skills deficiencies of the NF1 population to capture the phenotype with a more appropriate term than autism.

NF1 and Bone Health

Juha Peltonen. NF1 related lesions of bone can be primary, without any apparent reason other than NF1 itself, or secondary caused by adjacent tumor growths. Pseudarthrosis of long bones and dystrophic scoliosis usually manifest at a young age. A rapid and long lasting treatment is needed to treat these ailments and the outcome may fall far from full recovery. Systemic osseous abnormalities, low bone mineral density (BMD), are common in NF1. Osteoporosis is found in ~20–40% of adult patients with NF1 affecting both genders. A recent register-based study has shown that patients with NF1 have an increased fracture risk, especially, and in men and women aged ~40 or more, and in children with NF1. The increased fracture risk in adults with NF1 is associated with low BMD. Since NF1 has been associated with hyperactive Ras signaling and bisphosphonates partially affect Ras in osteoclasts, it may be possible the bisphosphonates have unexpected effects in patients with NF1. This is supported by the fact that osteoclasts derived from blood samples of patients with NF1 are insensitive to bisphosphonate-induced apoptosis in vitro compared to control osteoclasts. In our pilot study, five men and a woman, aged 28–76 years, with NF1-related osteoporosis were enrolled to the study. The medication included a weekly dose of 70 mg alendronate and a daily 20 μg vitamin D supplementation. After 23 months of follow-up, bone mineral density was increased in five out of six patients, but the increase was not statistically significant. Levels of serum bone turnover markers CTX and PINP were reduced, suggesting slower bone remodeling as expected. Even though the study group was small, the findings of the current study (one new fracture and one patient with decreased bone mineral density) call for a larger study to assess the efficacy of bisphosphonates in NF1-related osteoporosis. Our studies of craniofacial and dental manifestations in NF1 have revealed that periapical cemental dysplasia is common in women with NF1. The radiologic findings of this harmless condition may be mistakenly interpreted as infection requiring root canal treatment. Clinical examination of 110 patients showed that NF1 per se does not predispose to caries. The results are important to report since a common anecdotal perception is that the rate of caries may be higher in NF1 compared to reference population.

Overview of the RASopathies

Katherine A. Rauen. The RASopathies are a group of clinically related developmental disorders which are caused by germ-line mutations in genes that encode components, or regulators of the Ras/MAPK pathway. As a group, the RASopathies are one of the largest groups of malformation syndromes known, affecting approximately 1:1,000 individuals and include NF1, NS, NS with multiple lentigines, capillary malformation–arteriovenous malformation syndrome, CS, CFC and Legius syndrome. The Ras/MAPK pathway plays an essential role in the regulation of the cell cycle, differentiation, growth and cell senescence, all of which are critical to normal development. As a result, Ras/MAPK pathway dysregulation has been shown to have profound deleterious effects on both embryonic and later stages of development. Because the underlying molecular mechanism for these syndromes is dysregulation of the Ras/MAPK pathway, the RASopathies exhibit numerous overlapping phenotypic features, including reduced growth, characteristic facial features, cardiac defects, cutaneous abnormalities, neurocognitive delay and a predisposition to neoplasia, both benign and malignant.

Legius Syndrome

Eric Legius. Germline loss-of-function mutations in human *SPRED1* were first reported in 2007 in patients with a NF1-like phenotype (Legius syndrome), and since then several reports have confirmed this finding. Legius syndrome clinically presents with multiple café-au-lait spots with or without axillary or inguinal freckling. Other typical NF1 associated features (Lisch nodules, bone abnormalities, neurofibromas, optic pathway gliomas, and malignant peripheral nerve sheath tumors) are systematically absent. Loss-of-function mutations in *SPRED1*, a negative regulator of the RAS-MAPK pathway, result in overactivation of this signal transduction cascade. Recently a SPRED1 mutation database was established based on the Leiden Open Variation Database (LOVD) software and is accessible online at http://www.lovd.nl/SPRED1 The database contains a spectrum of different mutations: frameshift (32%), missense (32%), nonsense (22%), copy number changes (CNCs) (9%), splicing (2%), silent (1%), in-frame deletion (1%), and a mutation affecting the initiation codon (1%). Sixty-three mutations and deletions are definitely pathogenic, most likely pathogenic or suspected to be pathogenic, 7 *SPRED1* missense mutations are probably benign and 17 are still unclassified.

Costello Syndrome

Bronwyn Kerr. CS was first described in the 1970s by Jack Costello, a New Zealand Pediatrician, who recognized the key characteristics in two patients. There were no further publications until 1991 when der Kaloustian published a third case, suggested the name "Costello syndrome" and noted the resemblance to NS and CFC. Over the next decade, a number of reports defined the key clinical features: Severe feeding difficulty, in nearly all requiring nasogastric feeding or gastrostomy; mild to moderate intellectual disability; after infancy, a pleasant sociable personality; a distinctive face and hands; relative macrocephaly; short stature, in some due to growth hormone deficiency; warts at moist surfaces and a high incidence of cardiac abnormalities. A high risk of malignancy, particularly embryonal rhabdomyosarcoma in early childhood and bladder carcinoma from adolescence, emerged with recom-

mendations for a screening protocol. This was subsequently modified due to the observation, as yet unexplained, of abnormal urinary catecholamines as part of the CS phenotype. The discovery of activating mutations in *HRAS* established CS as a disorder of the Ras/MAPK pathway and illuminated the clinical overlap between CS, Noonan and CFC syndromes. Differentiation of CS from these disorders, particularly CFC, may be difficult, particularly in early life, as there are few if any clinical findings that are specific to one disorder rather than another. Mutation analysis has confirmed the core adult and childhood phenotypes, and permitted estimation of incidence, with a birth prevalence in the UK, based on mutation testing, from 2000 to 2009 of 1/381,914 live births. The prenatal phenotype has been delineated and a severe neonatal phenotype has emerged. Mild and atypical phenotypes have also been described. Severity scoring has demonstrated a genotype–phenotype correlation. A more accurate delineation of aspects of the natural history has been possible. A mouse model replicates some aspects of the human phenotype. These studies are a prerequisite for considering treatment trials, and accurate outcome measures. The views of affected patients and their families are however of at least equal importance in evaluating endpoints.

Capillary Malformation–Arteriovenous Malformation: A RASopathy Caused by RASA1 mutations

Miikka Vikkula. Capillary malformation–arteriovenous malformation (CM–AVM; OMIM 608354) is an autosomal dominantly inherited vascular disorder with high penetrance (97%) and variable expressivity. Affected individuals usually have small, multifocal, randomly distributed, pale capillary malformations, many of which have a white halo around. About one-third of the patients have an associated fast-flow lesion, which can involve the skin, subcutaneous tissue, muscles, bones, brain, or spine. The fast-flow lesion can be an arteriovenous malformation, or an arteriovenous fistula. The majority of them are located in head and neck, being extra- or intracranial. Intra-CNS fast-flow lesions are seen in about 10% of mutation carriers. Those in infants seem to be more severe and at higher risk of bleeding, including vein of Galen aneurysmal malformation. Spinal lesions also occur. Parkes Weber syndrome, characterized by a large capillary blush on an extremity, arteriovenous microfistulas, and bony and soft tissue hypertrophy, is also part of the phenotype. Those with CM–AVM, have additional CMs, distant to the Parkes Weber lesion. Some CM–AVM patients have cutaneous areas covered by numerous punctate telangiectasias with white halos. This may make one think of hereditary hemorrhagic telangiectasia. Occasionally, lymphedema is present in CM–AVM. CM–AVM is caused by loss-of-function mutations in *RASA1*. Over 100 heterozygous mutations have been identified in greater than 130 patients [(http://www.icp.ucl.ac.be/vikkula/VAdb/home.php) Most are private]. De novo mutations are frequent, occurring in about 30% of patients. Thus, familial history is not a diagnostic necessity. In about a fourth of patients, no coding region *RASA1* mutation can be identified. We have demonstrated for two other dominantly inherited vascular malformations that the inherited mutation is not sufficient for the multifocal lesions to develop. In mucocutaneous venous malformations (VMCM, OMIM 600195) due to a "weak" inherited gain-of-function *TIE2* mutation, a loss-of-function second-hit was identified in one lesion. In glomuvenous malformations (GVM, OMIM 138000), caused by inherited loss-of-function glomulin mutations, several second-hits have been identified. The most frequent is an acquired uniparental isodisomy (aUPID) of chromosome 1p, on which the glomulin gene is located. In CM–AVM we addressed this issue, by studying a likely radiotherapy-induced tumoral tissue that had arisen within a large CM on an arm. The tissue turned out to contain four mutations, two of which involved *RASA1*: An inherited splice site mutation and deletion of the complete long arm of chromosome 5, on which the *RASA1* is located. This combination should lead to complete local loss of p120RASGAP, encoded by *RASA1*. Thus, it is plausible that the double-hit mechanism is also involved in CM–AVM. We tested *RASA1* in two other disorders characterized by asymmetric limb enlargement and vascular malformations, namely in the Klippel–Trenaunay syndrome and in regional capillary malformation with overgrowth. We did not identify any clear pathogenic change in these patients. Thus, besides clinical and radiological criteria, *RASA1* testing constitutes an additional tool to differentiate patients with Parkes Weber syndrome of CM–AVM from clinically overlapping disorders. *RASA1* encodes p120RASGAP a RASGTPase. It is involved in turning down RAS activity after growth factor induced activation. In the absence of p120RasGap, murine cells show prolonged Ras signaling associated with defective migration. p120RasGAP−/− embryos die at E9,5, and mosaics generate overt superficial vascular defects by E15. Thus, modulators of RAS signaling pathway activity may hold the promise for future targeted therapies of CM–AVM, as for other RASopathies.

Cardio-facio-cutaneous Syndrome

Emma Burkitt-Wright. CFC is a severe condition that arises due to germ-line mutation in one of several genes of the Ras-MAPK pathway, most commonly *BRAF*. Such mutations nearly always arise de novo in the individual, and genetic and phenotypic overlaps with NS are well-recognized. Clinical features include developmental delay, feeding difficultsies, learning disability and epilepsy, congenital heart disease including pulmonary stenosis and atrial septal defect, hypertrophic cardiomyopathy and a wide range of ectodermal manifestations. Genotype–phenotype correlation is challenging, hampered by the small number of identified patients, extensive genetic and allelic heterogeneity and the fact that some patients have no identifiable mutation. We have recently identified a novel and strong association between *MAP2K1* p.Tyr130Cys mutations and extensive joint contractures, which have been progressive through childhood and early adult life, and a source of severe impairment in these patients. The existence of patients with a clinical CFC diagnosis and no identifiable mutation implies that further genes may be involved. All known genes for CFC and NS need to be tested before a novel causative gene can be invoked as the explanation for any such presentation. The identification of *SHOC2* mutations, initially described as the cause of a distinct presentation, "Noonan-like syndrome with loose anagen hair," in multiple patients with clinical diagnoses of CFC in the cohort identified in Manchester bears witness to this. Affected patients frequently had

severe and enduring feeding difficulties, often requiring gastrostomy, a variety of cardiac defects, short stature with relative macrocephaly, learning disability and a wide range of ectodermal manifestations, each of which were consistent with the clinical diagnosis of CFC.

Exome sequencing of patient-parent trios has power to identify de novo mutations, and hence to investigate the molecular basis of CFC. This approach identified a frameshift mutation in NF1 in a patient with a clinical diagnosis of CFC (on the basis of severe developmental delay, intractable feeding difficulties, short stature with relative macrocephaly, curly hair, and other ectodermal features), with a previous history of hepatoblastoma. While it is unclear if other factors may be contributory to this patient's phenotype, this observation provides proof of principle of the value of trio exome sequencing for investigating CFC, and highlights the overlapping nature of germ-line Ras/MAPK pathway disorders.

Noonan and NS with Multiple Lentigines

Michael A. Patton. I started my work on NS in 1986 after being invited to talk to the new UK patient group which was being set up. I did not know at that stage that I would be spending the next 25 years working on the syndrome, but it has been a very exciting journey and one of the highlights has been the opportunity to work with Dr. Jacqueline Noonan who described the syndrome in 1968. Around that time we set up a national clinical and genetic study on NS in the UK and we recruited around 150 families with whom we undertook a number of studies which I will describe. We were also able to keep in touch with this cohort of families and undertake a 20 year follow up study which we published. It is very important to understand the natural history of genetic disorders and I hope we will be able to revisit this cohort in the future as although we did not find an increase in the incidence of cancer there are still concerns that this may occur in later life as the genetic defect lies in a pathway with increased cellular growth. The facial features of NS change with age and can be quite subtle. We did a number of studies with facial measurement but worked with Peter Hammond in developing sophisticated photogrammetry software that can capture the facial features from children of different ages and "morph" through the different ages to demonstrate the changes. Such software does not provide a diagnosis on its own but has been very useful for training dysmorphologists about the changing facial phenotype. There have been many studies on the cardiac abnormalities in NS and the classic lesion is pulmonary stenosis. The heart valve is often thickened and the standard pull through valvotomy may not in some cases be sufficient. However encouragingly we found that 68% of the cohort only required follow up and did not require any surgical heart treatment. The growth studies confirmed that children with NS are on the lower centiles for height and may benefit from growth hormone. It is interesting to note that the head is relatively large and this is a feature that has been noted in other RASopathies without any specific mechanism being suggested. The hematological features of NS are diverse and still not fully explained by the underlying genetic mechanisms. The link between acute myelomonocytic leukemia and NS has been very elegantly dissected by Marco Tartaglio and colleagues. In our original studies we found that there are a variety of defects in the intrinsic coagulation cascade and in some cases there were defects in multiple coagulation factors which suggested that the genetic abnormality might be affecting the transcription of other genes in this pathway. To my knowledge defects of coagulation factors have not been reported in the other RASopathies. The diagnosis of NS has been greatly improved with molecular testing. There are now eight genes contributing to this disorder and testing individual genes has proven to be inefficient as it is now possible to use panels or next generation sequencing techniques to undertake this screen in a relatively short time frame. *PTPN11* mutation still account for around half of the cases of NS. The majority of cases of NS with multiple lentignes also have mutations in the *PTPN11* gene. The S*HOC2* gene is associated with NS with "loose anagen hair" or sparse slow growing hair and remains unique in having a specific genotype–phenotype correlation. The *RIT1*, a recently reported gene, was found in 17 out of 180 Noonan patients who have remained gene negative after conventional diagnostic testing. It is associated with a very high frequency of cardiomyopathy. Although there has been great progress in our understanding of this disorder there are still clinical features such as the joint pains and lymphedema that require more research and there are probably further genes to discover in the Ras/MAPK pathway. This has been a very stimulating meeting and there has been a useful cross-fertilization of ideas between the different approaches to this molecular pathway.

Animal Models and Treatment Trials for Noonan Syndrome

Martin Zenker. Cardiac abnormalities, short stature, and variable learning difficulties represent major clinical issues in NS and related RASopathies, and they are mainly responsible for the patients' need of long-term therapeutic intervention and medical care. While it is well established that constitutive Ras pathway dysregulation caused by mutations in several of its components or modulators is the common basis of this group of disorders, the precise pathophysiology that is underlying those clinical manifestations is incompletely understood. New insights have been gained from animal models for NS and have raised the idea of pathophysiology-based (causal) treatment for NS and related disorders. Various animal models for NS and related disorders, including mouse, zebrafish and fruitfly models, have been developed and studied during the last years. They have shown that activating mutations in the Ras/MAPK signaling cascade cause a characteristic pattern of cardiovascular, skeletal, and neurologic abnormalities in different species, recapitulating many aspects of the human disorders. Pharmacologic interventions have been explored to rescue phenotypic consequences of Ras pathway dysregulation in some animal models. These studies have been focused on cardiac, skeletal, and neurological manifestations of NS and their results can be summarized as follows: (i) Physical malformations such as heart defects and craniofacial abnormalities may be prevented by early (embryonal/prenatal) MEK inhibitor treatment; (ii) hypertrophic cardiomyopathy as an abnormality that is likely driven by ongoing dysregulation of intracellular signaling processes can be ameliorated or even reversed by postnatal inhibition of Ras/MAPK signaling

or related signaling pathways, such as PI3K-AKT, depending on the underlying genetic defect; (iii) as previously shown for Nf1 and Spred1 mouse models, inhibitor treatment may also be beneficial for learning issues in an animal model for NS. Together, those studies have also contributed to an understanding of Ras-dependent signaling as a complex signaling network where other Ras effector pathways besides the MAP kinases are involved, and may have to be considered in the development of new therapeutic strategies. Considering such complexity of physiological as well as disturbed signaling in those pathways, translation of the knowledge gained from animal studies into clinical practice is not trivial. No results of clinical trials using Ras pathway inhibitors have been published, so far. The only medical treatment for NS that has been explored in several studies is growth hormone treatment. General pharmacological considerations regarding inhibitor treatment for individuals affected by NS and related RASopathies have to include the following aspects: (i) Pharmacological inhibitors are not absolutely specific for distinct protein kinases; (ii) delivery of the drug to target tissues may grossly differ from each other (e.g., brain vs. heart); (iii) signaling happens in networks rather than linear pathways, and feedback loops may have to be considered; and (iv) restitution of a hypothetical basal pathway activity may not necessarily also restore physiological signaling dynamics. There is thus an obvious need of additional preclinical studies. On the way towards clinical trials, it will also be important to gain deeper knowledge about natural history and genotype–phenotype correlations in RASopathies. Family support groups need to be involved. Major practical questions that also remain to be answered are regarding the selection of appropriate study populations and pharmacological compounds (or a combination of compounds), parameters for monitoring of treatment effects, and the definition of treatment endpoints. Thus, despite the growing evidence that NS and related disorders may be amenable at least to some extent to small molecule inhibitor treatment, the most important thing we have to ask our patients for is to be patient.

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