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CLINICAL AND MOLECULAR CHARACTERIZATION OF NOONAN SYNDROME

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Preface

This thesis is a collection of several projects related to Noonan syndrome and Rasopathies I had the opportunity to develop myself or collaborate in development during my Ph.D. program. Rasopathies constitute an emerging group of clinically and genetically related disorders having RAS signaling dysregulation as shared pathogenic mechanism. Noonan syndrome is among the most common non-chromosomal disease affecting development and growth and represents a model of multisystemic developmental disorder. The projects presented aim at exploring different molecular and clinical aspects of this condition. The research areas of this dissertation range from a deepening of the molecular bases of this wide variable disorder to an expanding of the clinical phenotype, through the investigation of any correlation with prenatal signs. Up to date, our cohort consists of 103 patients with a molecularly confirmed diagnosis of a Rasopathie. The clinical diagnosis has been proposed according to the clinical criteria defined by van der Burgt [van der Burgt et al 1994]. Detailed clinical information were routinely collected from clinical records and anamnestic investigation. Table 1 and figure 1 summarize the genotypic spectrum of our patients.

Table 1 Genotypic spectrum of our cohort.

GENE	N° affcted individuals	Familial cases	Sporadic cases
PTPN11	70	9	46
SOS1	12	1	9
RAF1	9	2	4
SHOC2	4	-	4
BRAF	3	-	3
KRAS	1	-	1
MEK	1	-	1
RIT1	1	-	1
SOS2	1	-	1
HRAS	1	-	1

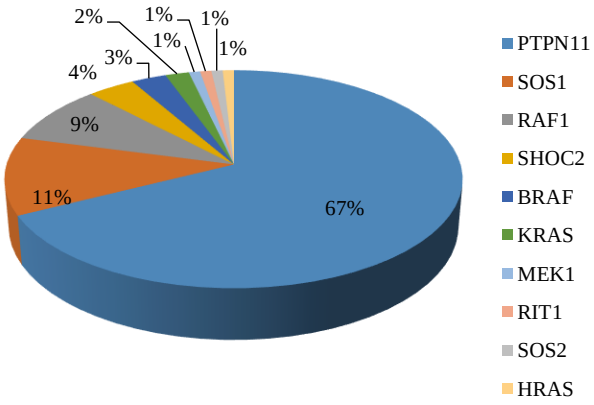

Fig. 1 Genotypic spectrum of our cohort.

SECTION I

INTRODUCTION

NOONAN SYNDROME

Noonan syndrome (NS, OMIM 163950) is a Mendelian autosomal dominant trait first described by the pediatric cardiologist Jacqueline Noonan more than 40 years ago [Noonan, 1968]. NS is thought to be relatively common, with an estimated prevalence of 1 in 1,000–2,500 live births, even if that estimate has never been confirmed on a population study. NS is characterized by a high clinical variability, ranging from cases with a very mild phenotype hard to be immediately recognized, to cases with a severe multisystemic involvement and typical facial and musculoskeletal dysmorphisms. Moreover, the phenotype becomes less pronounced with age [Allanson et al, 1985].

Clinical Features

The main clinical manifestations of NS include typical facial and skeletal dysmorphic appearance, congenital heart defects (CHD), postnatal reduced growth and cryptorchidism. Other relatively common features are ectodermal anomalies, bleeding diathesis, lymphatic dysplasias, and variable cognitive deficits. Because of this high clinical variability, in 1994 a scoring system was proposed by van der Burgt et al. to facilitate the clinical diagnosis [van der Burgt, 1994] (Fig.1)

Feature	A = Major	B = Minor
1 Facial	Typical face dysmorphology	Suggestive face dysmorphology
2 Cardiac	Pulmonary valve stenosis, HOCM and/or ECG typical of NS	Other defect
3 Height	<P3*	<P10*
4 Chest wall	Pectus carinatum/excavatum	Broad thorax
5 Family history	First degree relative with definite NS	First degree relative with suggestive NS
6 Other	Mental retardation, cryptorchidism and lymphatic dysplasia	One of mental retardation, cryptorchidism, lymphatic dysplasia

HOCM: hypertrophic obstructive cardiomyopathy;
*P3 and P10 refer to percentile lines for height according to age, with the normal range of variation defined as P3-P97 inclusive
Definitive NS: 1 "A" plus one other major sign or two minor signs; 1 "B" plus two major signs or three minor signs

Fig. 1 scoring system for NS. Adapted from van der Burgt et al. 1994

Facial and skeletal dysmorphisms – The main facial dysmorphisms includes broad and high forehead, hypertelorism, epicanthic folds, downslanting palpebral fissures, palpebral ptosis, low-set posteriorly rotated ears with a thick helix, flat nasal bridge, high arched palate and a short neck with excess nuchal skin and a low posterior hairline. The contour of the face appears triangular with age and the facial features becomes less pronounced (Fig. 2)

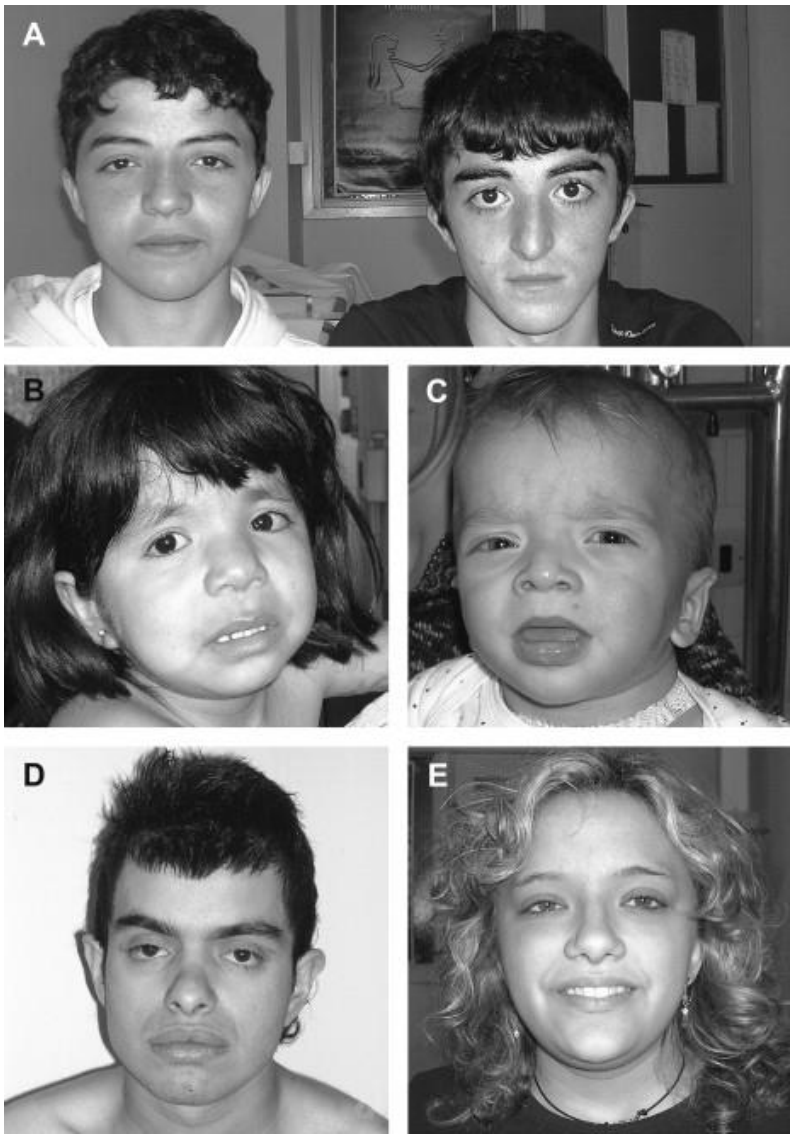

Fig.2 Facial appearance of six NS patients of our cohort. (A) two brothers, 16 and 18 years old, with particularly mild facial phenotype; (B) 2 years old female of South American ethnicity; (C) 2 years old boy; (D) 20 years old male; (E)16 years old female. [Ferrero et al. 2008]

Characteristic skeletal deformities consist of pectus carinatum and/or excavatum, broad thorax, scoliosis, cubitus valgus and joint hyperextensibility. Orthopedic problems are common in NS [Sharland et al. 1992] but are rarely severe enough to warrant surgical intervention. Giant cell lesions of the jaw, similar to those seen in cherubism, have been reported [Lee et al. 2005]. (Fig. 3)

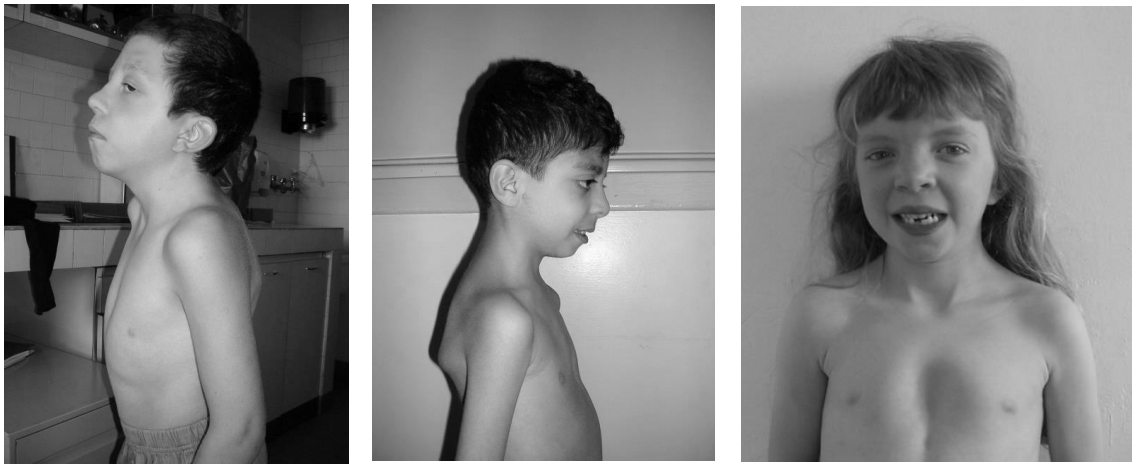

Fig. 3 Typical skeletal dysmorphisms in NS.

Congenital heart defects – Pulmonic stenosis (PS) and hypertrophic cardiomyopathy (HCM) are the most common cardiac defects described in NS, but a wide range of other lesions, including atrioventricular septal defects (AVSD), aortic coarctation and structural anomalies of mitral valve, are also observed. The most common congenital heart disease is pulmonary valve stenosis with dysplastic leaflets. The type and severity of the cardiac disease can vary from minor and inconsequential forms to life- threatening forms [Burch et al. 1993; Marino et al. 1999; Bertola et al. 2000].

Growth pattern- Short stature is one of the striking features of NS**,** with median heights under the third centile in affected children. Noonan-specific growth curves were designed [Witt et al., 1986; Ranke et al., 1988]. (Fig. 4)

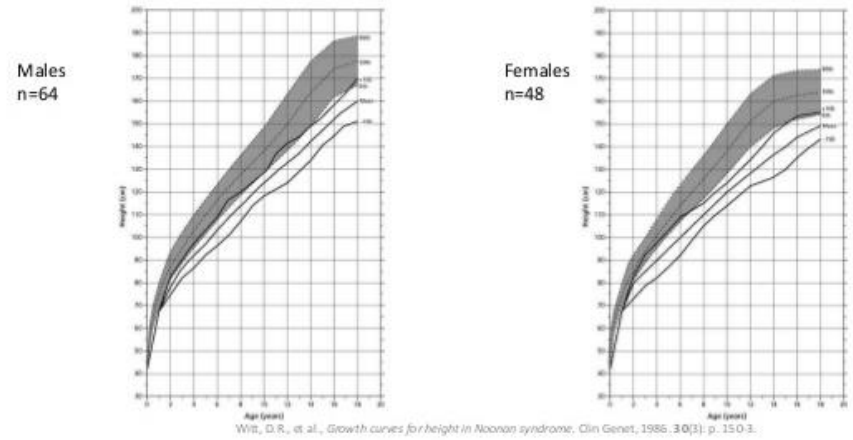

Fig. 4 Noonan specific growth charts, by Witt et al. 1986.

At birth, weight and length are usually normal, birth weight can be high due to the perinatal lymphedema. Mean postnatal growth is often under the third centile for height and weight. Onset of puberty and average bone age are delayed by approximately two years and the pubertal growth spurt is frequently reduced or absent. Several studies of growth hormone (GH) secretion in NS have documented some quantitative and qualitative abnormalities [Ahmed et al., 1991; Tanaka et al., 1992; Noordam et al., 2002]. Decreased insulin growth factor I (IGF-I) together with low responses to provocation, suggest impaired GH release, or disturbance of the GH/IGF-I axis. Mild growth hormone resistance related to a post-receptor signaling defect, which may be partially compensated by elevated growth hormone secretion, is also reported [Allanson et al. 2016]. It was largely investigated the potential effects of GH therapy during childhood. A prospective survey on the effect of long-term GH treatment on adult height in NS documented a significant mean height gain [Noordam et al., 2008]. Similarly, another study indicated that GH might significantly improve height in children with NS with earlier initiation of therapy [Romano et al., 2009].

Lymphatic dysplasia – Lymphatic vessel dysplasias are common findings in NS, although the persistence of lymphedema at birth is not constant. Lymphatic dysplasia lead to generalized or peripheral lymphedema and intestinal or pulmonary lymphangiectasia, leading to protein-losing enteropathy or spontaneous chylothorax. Varying degrees of edema or fetal hydrops are often present during intrauterine life, frequently revealing a cystic hygroma in early pregnancy. This feature successively regresses and may result in excess nuchal skin and pterygium colli after birth. Chylothorax may occur in childhood spontaneously or as a complication of cardiac surgery [Bottner et al. 2005].

Hematological disorders - Bleeding diathesis frequently occurs in patients with NS, especially in childhood. Nearly 40% of patients with NS had a bleeding diathesis and >90% of them had platelet function and/or coagulation abnormalities [Artoni et al. 2014]. Coagulation studies revealed von Willebrand disease, deficiencies of factors XI and XII, thrombocytopenia and platelet function defects [Sharland et al., 1992b; Massarano et al., 1996; Singer et al. 1997]. Since many affected children undergo one or more interventional procedures, in particular orchidopexy, special care is required to prevent intraoperative or postoperative hemorrhagic complications. Moreover, NS patients are at increased risk of develop myeloproliferative disorders (MPD), in particular juvenile myelomonocytic leukemia (JMML)**.** MPD in children with NS (NS/JMML) may regress without treatment, follow an aggressive clinical course or evolve to acute myeloid leukemia [Bader-Meunier et al. 1997; Fukuda et al. 1997; Choong et al. 1999], however the prognosis for NS patients with JMML is better than that for non-syndromic JMML.

Neuropsychomotor development - Feeding problems occurs in the majority of NS affected infants and can cause failure to thrive [Shah et al. 1999]. In most patients, feeding difficulties resolve by around age 18 months. Overall, developmental delay and learning disabilities are quite common in NS, affecting one-third of patients [Sharland et al. 1992]. Children with NS could demonstrate mild motor dela**y**, which may be partly subordinate to the muscular hypotony that is often present in early childhood. Achievement of developmental milestones is deferred: mean age for sitting is 10 months, that for walking alone is 21 months, and that for talking is 31 months [Sharland et al. 1992]. Social problems and attention deficit have also been observed; of note, alexithymia in adult subjects with NS has also been reported [Verhoeven et al. 2008].

Molecular Basis

NS is a Mendelian trait transmitted in an autosomal dominant manner. It is genetically heterogeneous, which partially explains its remarkable clinical variability. Up to date, heterozygous mutations of several functionally related genes have been identified (see below). All these genes encode for proteins involved in the RAS/mitogen activated protein kinase (RAS/MAPK) signal transduction cascade, which has been documented to play a keyrole in developmental processes and growth. Mutations of these genes all lead to deregulated, generally enhanced signal flow through RAS-mediated signal transduction cascades. The Ras proteins are small guanosine nucleotide-bound GTPases that act as a central hub for numerous intracellular signaling pathways. They switch between an active GTP-bound and an inactive GDP-bound form, through the binding of growth factors to the receptor tyrosine kinases (RTKs), causing the RTK autophosphorylation and its interaction with downstream effectors (Fig. 5).

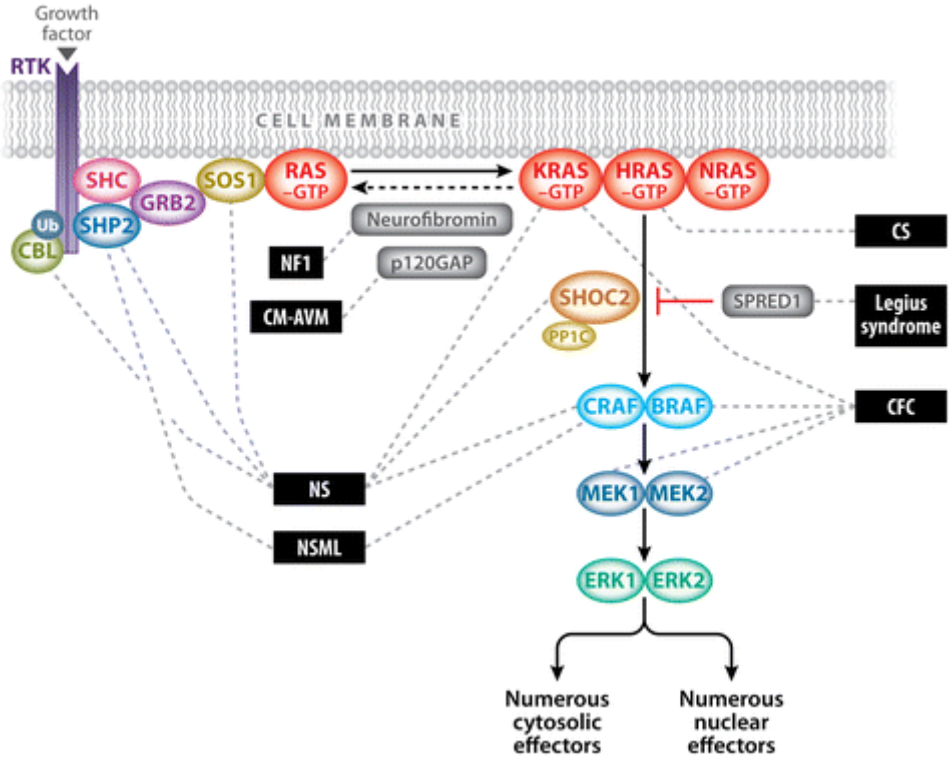

Fig. 5 The RAS-MAPK signal transduction pathway. Rauen et al. 2013

The RAS-MAPK cascade is activated in response to different extracellular stimuli, such as cytokine, hormone and growth factors, and mediates different biological functions such as proliferation, migration, survival, differentiation and senescence .It is a major mediator of early and late developmental processes, including morphology determination, organogenesis, synaptic plasticity and growth. This pathway has also been largely investigated in oncogenesis since somatic mutations occurs in several neoplasms. However, biochemical studies have demonstrated that the novel germline mutations identified in NS and related disorders are not as strongly activating as those associated with tumors, likely due to the embryonic lethality arising from these germline mutations [Kratz et al. 2005, Tartaglia et al. 2006]. The most common gene associated with NS is PTPN11, which accounts for approximately 50% of all cases [Tartaglia et al. 2001]. SOS1 is the second-most-common gene responsible of NS, accounting for approximately 15% of cases [Roberts et al. 2007, Tartaglia et al.

2007]. SOS1 encodes the Ras guanine nucleotide exchange factor (RasGEF), which is responsible for stimulating the conversion of Ras from the inactive GDP-bound form to the active GTP-bound form. Mutations in RAF1 also cause NS [Pandit et al. 2007, Razzaque et al. 2007]. RAF1 encodes the protein RAF1, a serine/threonine kinase that is one of the direct downstream Ras effectors. KRAS mutations are a rare cause of NS [Shubbert et al. 2006]. KRAS mutations increase signaling of the Ras/MAPK pathway through two distinct mechanisms: reducing the intrinsic GTPase activity or interfering with the binding of KRAS and guanine nucleotides. Mutations in NRAS have also been identified in a very small percentage of NS [Cirstea et al. 2010]. In 2013 Aoki et al. identified germline mutations in RIT1, a widely expressed small GTPase belonging to a subfamily of the RAS family, which gain of function mutations accounts for approximately 5% of cases of NS [Aoki et al.2013, Cavè et al. 2016]. Recently, mutations in RRAS [Flex et al. 2014], RASA2 [Chen et al. 2014], *SOS2* [Cordeddu et al. 2015], and LZTR1 [Yamamoto et al. 2015] have added further heterogeneity to the NS scenery. More recently, an autosomal recessive form of NS has been documented, due to a biallelic mutations in LZTR1 [Jhonston et al. 2018]. A few years ago, two conditions clinically resembling NS have been described as being caused by mutations in *CBL* and *SHOC2*, respectively. Loss of function *CBL* mutations account for <1% of cases of NS, especially when juvenile myelomonocytic leukemia (JMML) occurs [Martinelli et al. 2010; Niemeyer et al. 2010]. CBL encodes an E3 ubiquitin ligase that negatively regulates the Ras/MAPK signaling downstream of RTK [Dikic et al. 2010]. Because mutations in CBL reduce the turnover of activated RTK, they have an overall effect of increased ERK activation. The SHOC2 missense mutation c.4A > G (p.Ser2Gly) underlies a disorder originally termed "Noonan-like syndrome with loose anagen hair. SHOC2 is an ubiquitously expressed scaffold

protein in the ERK1/2 pathway, which ties RAS, RAF-1 and the catalytic subunit of protein phosphatase 1c (PP1c). Recruitment of PP1c enables dephosphorylation of the S259 residue of RAF-1 resulting in the hyperactivation of RAF-1 [Rodriguez-Viciana, et al., 2006]. Recently a novel mutation in SHOC2(c.519G>A; p.M173I) has been reported, leading a phenoype partially overlapping those occurring in NS [Hannig et al. 2014].

The Rasopathies

The Rasopathies are a class of developmental disorders, including NS, characterized by constitutional deregulation of the RAS-MAPK pathway and caused by germline mutations in several genes encoding components or regulators of the pathway. Because the underlying molecular mechanism for these syndromes is the same, these entities share many clinical features, including facial dysmorphisms, a wide spectrum of CHD, postnatal growth failure, variable degrees of neurocognitive impairment, skeletal and ectodermal anomalies, and increased tumor risk [Tydiman et al. 2009, Digilio et al. 2011, Rauen et al. 2013, Rauen et al. 2015, Aoki et al. 2016]. Taken together, the Rasopathies represent one of the most prevalent groups of congenital malformation syndromes affecting approximately 1 in 1,000 individuals [Rauen et al. 2013]. Besides NS, this group includes Neurofibromatosis type 1, Costello syndrome, Cardiofaciocutaneous syndrome, Legius syndrome and NS with multiple lentigines (previously referred to as LEOPARD syndrome).

Neurofibromatosis type 1- NF1 is an autosomal dominant disorder affecting approximately 1 in 3,000 newborns [Williams et al 2009]. The clinical diagnosis of NF1 is based on the presence of the following criteria: café-au-lait maculae,

intertriginous freckling, neurofibromas and plexiform neurofibromas, iris Lisch nodules, osseous dysplasia, optic pathway glioma, and/or a first-degree relative with NF1. NF1 patients show dysmorphic craniofacial features reminiscent of NS [Huffmeier et al. 2006] mild neurocognitive impairment, and a predisposition to developing certain malignancies. In particular pediatric malignancies include optic pathway glioma, rhabdomyosarcoma, neuro-blastoma, and juvenile myelomonocytic leukemia, whereas adult malignancies include malignant peripheral nerve sheath tumors, gastrointestinal stromal tumors, pheochromocytomas, and breast cancer. NF1 is caused by mutations in the NF1 gene, with approximately half of the mutations being de novo [Cawthon et al. 1990, Wallace et al. 1990]. NF1 encodes for neurofibromin, a GTPase-activating protein that is a negative regulator of Ras. NF1 mutations result in neurofibromin loss of function, causing reduced RasGTPase activity and resulting in an overall increase in active GTP-bound Ras. Segmental and mosaic forms and gonadal mosaicism are described.

Costello syndrome (CS) – CS is one of the rarer Rasopathies. Craniofacial dysmorphisms are typical and include relative macrocephaly, coarse face, full lips, large mouth, full nasal tip, curly or sparse fine hair, epicanthal folds and wide nasal bridge. Other key features are severe failure to thrive especially in the newborn period, short stature, cardiac anomalies, musculoskeletal and ectodermal abnormalities, hypotonia and mild to moderate intellectual disability [Rauen et al. 2007]. Phenotypic features become apparent in the perinatal period with polyhydramnios, prematurity and increased birth weight. (Fig. 6)

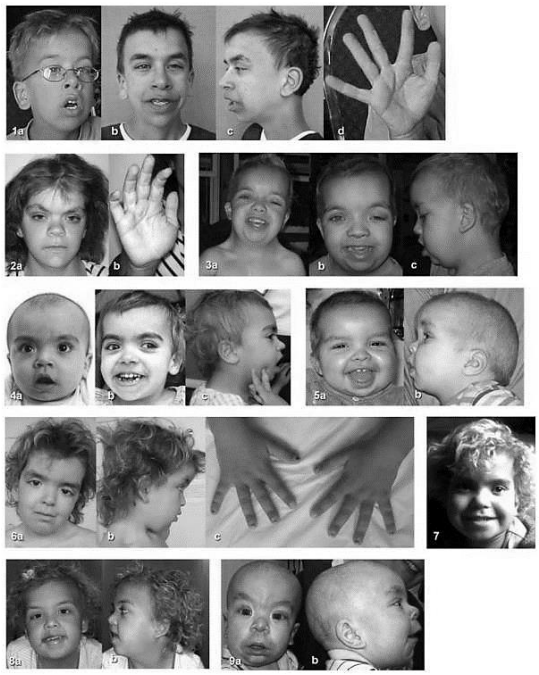

Fig. 6 Facial phenotype of CS. From Gripp et al. 2011

A high risk of malignancy, both benign and malignant, is described, including benign cutaneous papillomas [Siegel et al 2011], embryonal rhabdomyosarcoma, transitional cell carcinoma, and neuroblastoma [Gripp et al 2005]. CS is caused by heterozygous activating germline mutations in HRAS [Aoki et al. 2005]. HRAS mutations cause a reduction in intrinsic and GAP-induced GTPase activity, resulting in Ras hyperactivation. Somatic mosaicism, autosomal dominant transmission [Sol-Church et al. 2009] and gonadal mosaicism [Gripp et al. 2011] have been anecdotally documented in CS [Gripp et al. 2006].

Cardiofaciocutaneous syndrome (CFC) – CFC is a quite severe condition, which genetic and phenotypic features overlap with NS. Affected patients present short stature, a wide spectrum of cardiac defects, with the most prevalent being PS, septal defects and HCM, severe and durable failure to thrive, a varied range of ectodermal

anomalies, and neurological manifestations including hypotonia, seizures, motor and speech delay, and/or learning disability [Yoon et al. 2007]. CFC individuals have NS-like facial dysmoprhisms, comprising macrocephaly, a broad forehead, bitemporal narrowing, hypoplasia of the supraorbital ridges, down-slanting palpebral fissures with ptosis, a short nose with a depressed nasal bridge and low-set, posteriorly rotated ears. Ectodermal findings are distinctive of this condition and consist of sparse, curly hair with sparse eyebrows and eyelashes, hyperkeratosis and keratosis pilaris. [Siegel et al. 2011] (Fig. 7).

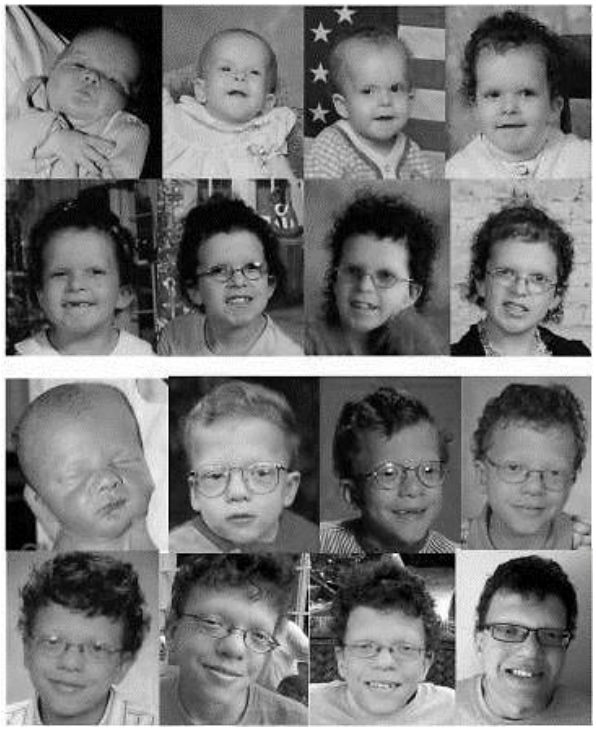

Fig. 7 Facial phenotype of CFC syndrome. From Pierpont et al. 2014

Four genes encoding for proteins of the Ras/MAPK pathway have been associated with CFC syndrome: BRAF, MAP2K1 (MEK1) and MAP2K2 (MEK2) and KRAS [Rodriguez et al. 2006]. Heterozygous BRAF mutations are found in approximately 75% of mutation-positive CFC individuals. BRAF is a serine/threonine protein kinase

and one of the direct downstream effectors of Ras. Heterozygous missense mutations in MAP2K1 (MEK1) and MAP2K2 (MEK2) are present in approximately 25% of CFC individuals. MEK1 and MEK2 are threonine/tyrosine kinases having the ability to phosphorylate and activate ERK1 and ERK2 [Tidyman et al. 2009].

Noonan syndrome with multiple lentigines (NSML) – NSML (previously referred to as LEOPARD syndrome) is a rare autosomal dominant disorder. LEOPARD was an acronym for the initial letters of the characteristic symptoms: lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic valve stenosis, abnormal genitalia, retardation of growth and deafness [Gorlin et al. 1971]. NSML and NS are allelic disorders, caused by different heterozygous missense mutations in the PTPN11 gene [Digilio et al. 2002, Legius et al. 2002] and RAF1 [Pandit et al.2007].

Legius syndrome (LS) - LS is an autosomal dominant RASopathy sharing many phenotypic features with NF1. Affected individuals may have dysmorphic craniofacial features reminiscent of NS, café-au-lait maculae, axillary or inguinal freckling and mild neurocognitive impairment. Contrary to NF1, this syndrome do not seem to be associated with a high risk of benign and malignant neoplasms. LS is caused by germline loss-of-function mutations in SPRED1 gene, a negative regulator of the RAS-MAPK pathway, resulting in hyperactivation of this cascade [Brems et al. 2007].

SECTION II

INVESTIGATIONAL CONTRIBUTIONS

Chapter 1.

Deepening the molecular basis and their consequences in Noonan syndrome: Characterization of a Novel PTPN11 Mutation Cluster

This work has been published as

Pannone L, Bocchinfuso G, Flex E, Rossi C, ***Baldassarre G****, Lissewski C, Pantaleoni F, Consoli F, Lepri F, Magliozzi M, Anselmi M, Delle Vigne S, Sorge G, Karaer K, Cuturilo G, Sartorio A, Tinschert S, Accadia M, Digilio MC, Zampino G, De Luca A, Cavé H, Zenker M, Gelb BD, Dallapiccola B, Stella L, Ferrero GB, Martinelli S, Tartaglia M.Structural,* **Functional, and Clinical Characterization of a Novel PTPN11 Mutation Cluster Underlying Noonan Syndrome.** *Hum Mutat. 2017 Apr;38(4):451-459*

BACKGROUND

In 2001 PTPN11 (MIM# 176876) was the first gene identified as responsible for NS [Tartaglia et al. 2001]. It encodes for the ubiquitously expressed non-receptor Src-homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2), which is implicated in multiple intracellular signaling pathways, including the RAS-MAPK and PI3K-AKT cascades [Tartaglia et al. 2004a.; Tajan et al. 2015]. SHP2 contains two tandemly arranged *N*-terminal SH2 domains (N-SH2 and C-SH2), a catalytic PTP domain and a *C*-terminal tail with a still largely uncharacterized function. SHP2's activity and subcellular localization is controlled by an allosteric switch involving the N-SH2 and PTP domains [Hof et al., 1998]. Under basal conditions, the catalytically inactive conformation of the phosphatase is stabilized by a wide intramolecular binding network involving residues located at the N-SH2/PTP interface. Binding of phosphotyrosine (pY)-containing signaling partners at a different site of the N-SH2 domain promotes the release of this autoinhibitory interaction, making the catalytic site available to substrates. Germline mutations of PTPN11 account for approximately 50% of cases of NS. PTPN11 mutations have been classified into six major groups [Tartaglia et al., 2006]. Groups I and II comprise lesions affecting the N-SH2/PTP interface, participating (group II) or not (group I) in catalysis. Changes belonging to group III and IV affect residues mediating substrate specificity or with a role in maintaining the overall PTP structure, respectively. Group V mutations involve residues located at the binding cleft of each SH2 domain. Finally, group VI includes lesions affecting amino acids located within the linker stretch connecting the N-SH2 and C-SH2 domains. (Fig. 1.1)

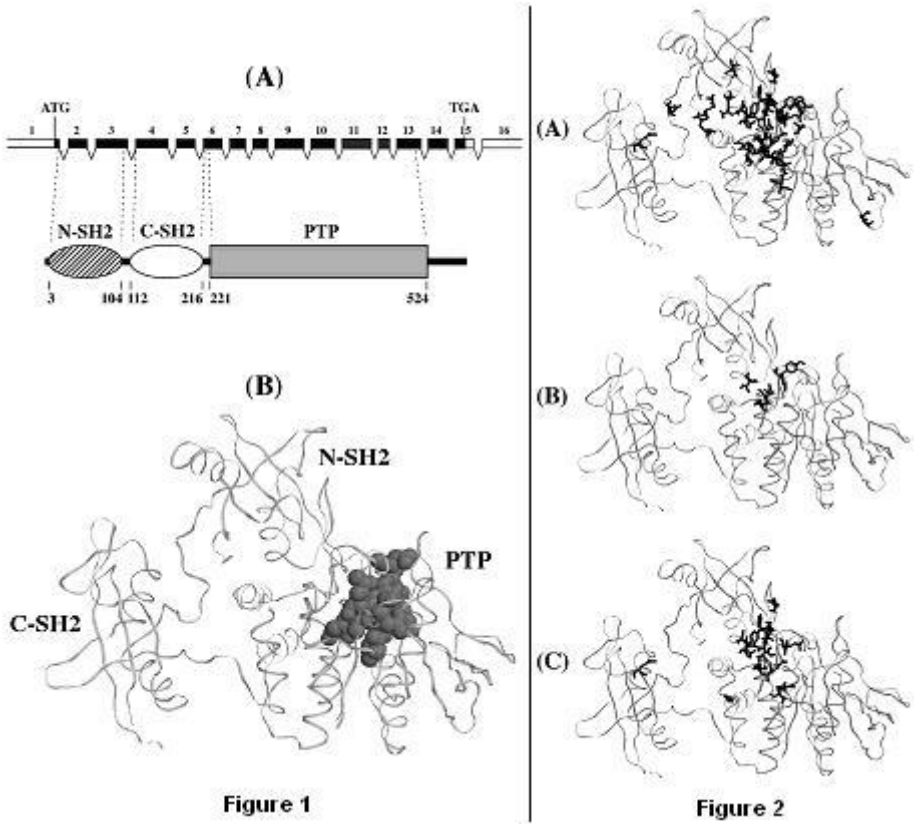

Figure 1.1 : (A) The PTPN11 gene and SHP-2 domain characterization. The coding exons are shown as numbered filled boxes. The functional domains of the protein, comprising two tandemly arranged SH2 domains at the N terminus (N-SH2 and C-SH2) followed by a protein tyrosine phosphatase (PTP) domain, are shown below. Numbers below the domain structure indicate the amino-acid boundaries of those domains. From Tartaglia et al. 2001 (B) Three-dimensional structure of SHP-2 in its catalytically inactive conformation, as determined by Hof et al. (1998). Residues involved in catalysis are shown (space fill). Figure 2 : Location of SHP-2 mutated residues in human disease. [Tartaglia and Gelb 2005]

A group of somatic mutations of PTPN11 occurs in childhood myeloproliferative and myelodysplastic disorders, as well as leukemias [Tartaglia et al. 2003, 2004, 2006]. Most mutations underlying NS or contributing to hematologic malignancies cluster at the N-SH2/PTP interface. These activating lesions are known to destabilize the autoinhibitory interactions between these two domains, enhancing basal phosphatase activity and affinity for binding partners [Keilhack et al., 2005; Tartaglia et al., 2006]. In contrast, NSML-associated mutations dramatically affect the catalytic activity of the enzyme [Keilhack et al., 2005; Hanna et al., 2006; Kontaridis et al., 2006; Tartaglia et al., 2006; Martinelli et al., 2008], and promote enhanced PI3K-AKT signaling

[Edouard et al. 2010]. The effects of mutations on the functional regulation of SHP2, however, can be more complex [Martinelli et al. 2012], and the precise structural and functional consequences of many of these lesions remain to be elucidated. This work reports on a novel PTPN11 mutation cluster causing NS and the associated clinical phenotype.

PATIENTS AND METHODS

Patients - Mutation analysis of *PTPN11* was performed in a diagnostic setting in a cohort of subjects with a clinical diagnosis of NS. Clinical assessment was performed by experienced pediatricians and clinical geneticists. DNA samples and clinical data were collected following institutional review board-approved protocols, and written informed consent for genetic analyses was obtained from all patients.

Molecular Data - Genomic DNA was isolated from circulating leukocytes by using standard techniques. The entire *PTPN11* coding sequence, together with the exon/intron boundaries and their flanking intronic regions were scanned for mutations by either Sanger sequencing or parallel targeted resequencing. Sanger sequencing used ABI BigDye Terminator Sequencing chemistry (Applied Biosystems, Foster City, CA, USA) and ABI 3700/3500 Capillary Array Sequencers (Applied Biosystems). Massive parallel resequencing was performed on MiSeq (Illumina, San Diego, CA, USA) and PGM (Life Technologies, Carlsbad, CA, USA) platforms, using different custom panels designed to target coding exons and flanking intronic sequences of most of the known NS/NSML genes, as well as genes causing clinically related phenotypes (i.e.,

PTPN11, CBL, SOS1, SHOC2, NF1, SPRED1, KRAS, HRAS, NRAS, RAF1, BRAF, MAP2K1, MAP2K2, and RIT1).

RESULTS

Among approximately 1,500 subjects with clinical diagnosis of NS or NSML screened in centers participating in the NSEuroNet Consortium, found to carry heterozygous mutations in *PTPN11*, five different missense changes affecting residues Leu^{261}, Leu^{262}, and Arg^{265} were identified in 16 unrelated individuals (NS EuroNet database, https://nseuronet.com/php/index.php). These variants accounted approximately for 1% of cases, specifying a novel mutation cluster causing NS. All mutations were novel, with the exception of the c.781C > T transition (p.L261F) that had previously been reported in one NS patient [Ezquieta et al., 2012]. This lesion, as well as the c.785T > G (p.L262R) and c.794G > A (p.R265Q) changes were shown to occur as de novo events by genotyping of parental DNAs in seven cases, while they co-segregated with the disease in four families. Similarly, the c.782T > A (p.L261H) and c.784C > T (p.L262F) substitutions co-segregated with the trait in single families. The c.781C > T and c.794G > A transitions were also found to co-occur as de novo events in one patient. All variants were considered to be pathogenic for the following reasons. First, they arose as de novo events in several patients. Second, they were not reported in public databases or occurred with a frequency below 1/20,000 in the Exome Aggregation Consortium's database (ExAC; http://exac.broadinstitute.org). Third, they were non-conservative, affected highly conserved residues among PTPN11 orthologs in vertebrates. Finally, the three affected residues are located in close spatial proximity to residues previously identified to be mutated in NS (i.e., Gln^{256} and

Gly^{268}). Based on these considerations and their biochemical/functional impact in vitro, all changes but c.782T > A (p.L261H) satisfied the criteria to be considered pathogenic according to the ACMG guidelines [Richards et al. 2015].

Phenotypic Spectrum Associated with Mutations at Codons 261, 262, and 265

Extensive clinical data were available for all probands and four affected relatives. Genotype–phenotype correlation analyses revealed that lesions affecting residues 261, 262, and 265 are associated with a relatively variable phenotype within the NS phenotypic spectrum. Remarkably, clinical features were quite subtle in the majority of cases. In particular, we noticed a significant lower prevalence of cardiac defects compared with what observed among PTPN11 mutation-positive patients (9/19 vs. 236/285, $P < 0.001$; two-tail Fisher's exact test) and the general NS population (132/151, $P < 0.001$) [Sarkozy et al., 2009]. Restricting our analysis to NS cases with *PTPN11* mutations, we recorded a lower prevalence of pulmonary valve stenosis, which occurred only in 37% of cases (7/19 vs. 247/362, $P < 0.02$). None of these patients exhibited HCM. A relatively low prevalence of short stature and less evident typical facial features were also observed. Finally, no significant cognitive and behavioral anomalies were reported in the subset of subjects carrying a mutation at residue Leu^{261}.

DISCUSSION

Approximately half of NS cases is caused by mutations in PTPN11. Multiple classes of PTPN11 mutations with a distinct perturbing effect on SHP2's function have been identified. This work reports on the identification of a novel cluster of germline

missense mutations in *PTPN11* underlying NS. The new mutation cluster affects a region of the PTP domain of SHP2 involved in inter- and intra-domain interactions. The collected data documented an heterogeneous impact of mutations involving these adjacent residues on protein function and intracellular signaling, which was, however, associated with an overall relatively mild phenotype. Similar to the majority of PTPN11 mutations causing NS and leukemia, substitutions at residues Leu^{262} and Arg^{265} affect the N-SH2/PTP interacting surface and enhance SHP2's function by perturbing the autoinhibitory conformation. Consistent with that, the collected biochemical data support the idea that mutations at codons 262 and 265 behave as typical group I lesions. The structural and biochemical data suggest that the missense changes affecting Leu^{261} belong to group IV and likely exert their pathogenic effect by perturbing SHP2's function at different levels. Leu^{261} is part of a hydrophobic core including Phe^{285} and Arg^{498}, which are in tight contact with residues surrounding the active site of the protein that play a key role in controlling substrate specificity [Andersen et al., 2001], and whose mutations cause NS and NSML, respectively [Tartaglia et al. 2006]. Based on their consequences on SHP2's structure and function, their variable activating effect on the MAPK signaling cascade, and their de novo origin in several instances, most changes affecting the novel mutation cluster can be considered as *bona fide* mutations causing NS. Among these, however, the missense change predicting the p.L261H substitution was identified only in one familial case (Fig. 1.2).

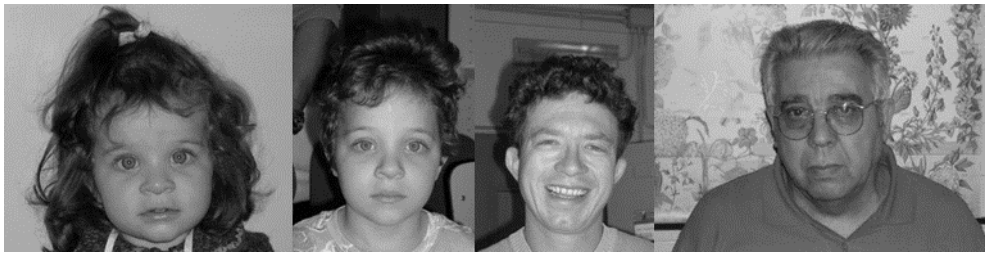

Fig. 1.2 The Leu^{261} familial case. The mutation was identified in the two probands, their father and their grand-father. Of note the mild facial phenotype, only suggestive of NS. Both sisters presented PS and short stature, while the affected relatives suffered of cryptorchidism in childhood.

For this variant, the evidence provided by the in silico data was not unambiguously supported by the experimental data, which were indicative of enhanced activity of the mutant only at high phosphopeptide concentrations in vitro, and demonstrated a significant upregulation of signaling through the MAPK cascade in transiently transfected cells only when they were treated with high doses of EGF. Based on these considerations, we deem the c.781C > T substitution as a functionally relevant change with mild clinical impact, even though, in the absence of any additional evidence supporting pathogenicity, this change should formally be classified as a variant of unknown significance. The clinical features associated with PTPN11 mutations at codons 261, 262, and 265 undoubtedly fall within the NS phenotypic spectrum. Genotype–phenotype correlation analysis revealed that affected subjects exhibit a relatively lower prevalence of cardiac defects (47%) compared with both PTPN11-positive (83%) and PTPN11-negative (64%) NS populations [Sarkozy et al. 2009]. Among individuals with a mutation affecting those residues, a significantly lower prevalence of PS compared with what generally observed among NS patients with PTPN11 mutations was observed (37% vs. 68%, $P < 0.02$). A major finding also regarded the relatively lower prevalence of short stature/length below the third centile in these subjects (60%) compared with PTPN11 mutation-positive NS cases (76%–93%) [Sarkozy et al. 2009; Roberts et al. 2013]. Finally, a mild phenotype was

particularly noted in subjects heterozygous for mutations affecting Leu^{261}. This genotype–phenotype correlation seems to be consistent with the apparently moderate functional impact of mutations involving that residue.

Chapter 2.

Phenotypic variability: the paradigm of the SHOC2 c.4A>G missense mutation

This work has been published as

***Baldassarre G**, Mussa A, Banaudi E, Rossi C, Tartaglia M, Silengo M, Ferrero GB.* Phenotypic variability associated with the invariant SHOC2 c.4A>G (p.Ser2Gly) missense mutation. *Am J Med Genet A. 2014 Dec;164A(12):3120-5*

BACKGROUND

NS is characterized by a high clinical variability, ranging from very mild to severe phenotype. Two conditions clinically resembling NS have recently been described as being caused by mutations in CBL and SHOC2, respectively. The apparently invariant SHOC2 missense mutation (c.4A > G), predicting the p.Ser2Gly amino acid substitution, underlies a RASopathy originally termed "Noonan-like syndrome with loose anagen hair (NS/LAH, OMIM 607721)", characterized by facial dysmorphisms resembling NS, growth retardation, cardiac anomalies (in particular dysplasia of the mitral valve and septal defects), and variable neurocognitive impairment [Mazzanti et al. 2003; Cordeddu et al. 2009]. A unique feature of NS/LAH is its association with easily pluckable, slow growing, sparse, and thin hair. Despite the clinical features associated with the c.4A > G change define a distinctive and relatively homogeneous phenotype, recent reports have provided first evidence for significant phenotypic variability [Capalbo et al. 2012; Hoban et al. 2012; Gripp et al. 2013]. Here we report on two additional cases of molecularly confirmed NS/LAH characterized by extremely different phenotypes.

PATIENTS

Patient 1- The proband was the third child of apparently healthy and non-consanguineous parents of Italian origin, with unremarkable family history. He was born at 38 weeks of gestation by caesarean after a pregnancy characterized by increased nuchal translucency and normal CVS karyotype (46, XY). The fetal ultrasound at 20th week of gestation revealed a cardiac defect consisting of a left

superior vena cava in coronary sinus associated with mild tricuspid valve incompetence. Birth weight was 3,160 g (50th centile), birth length was 47 cm (50th centile), and birth OFC was 34 cm (50–75th centile). Apgar scores were 9 and 10 at 1 and 5 min, respectively. A preliminary physical examination revealed a cardiac murmur, cryptorchidism, and mild dysmorphisms including neck skin redundancy, low set and posteriorly angulated ears, hypertelorism, and flat nasal bridge (Fig. 2.1)

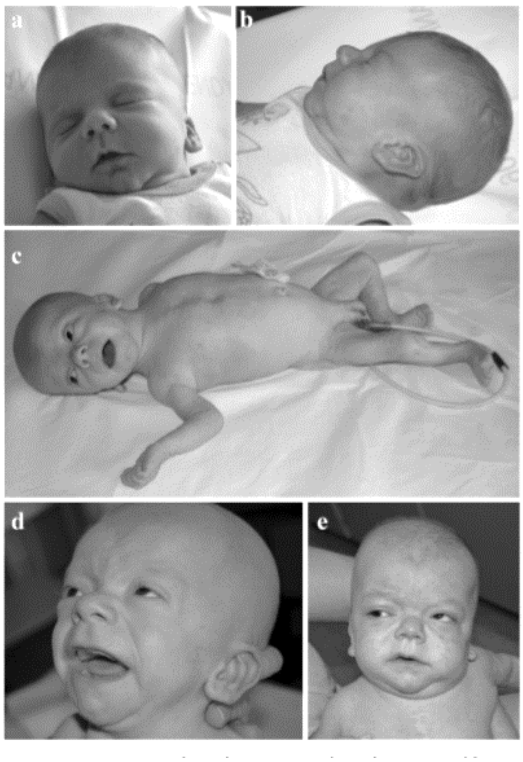

Fig. 2.1 Patient 1 at birth (a and b), at age 5 months (c and d), and 6 months (e)

An echocardiogram performed at birth showed a dysplastic mitral valve with redundant tissue, a single medial-posterior papillary muscle, a perimembranous ventricular septal defect with a left-to-right shunt, moderate septal hypertrophy and a mild dysplastic tricuspid, and semilunar valve. Beta-blocking therapy was initiated, with a partial control of the subaortic gradient. Electrocardiogram (ECG), abdominal

ultrasound (US), and echoencephalography were normal. Neurologic performances in the first weeks of life were adequate for age. The association of congenital heart disease with mild dysmorphisms, allowed to suggest a diagnosis of NS, but no mutations in the entire coding sequence of the PTPN11, SOS1, RAF1, KRAS, and RIT1were identified. At age 2 months, he was admitted for heart failure manifesting with tachypnea, profuse sweating, and feeding problems with weight loss. Enteral nutrition by gavage was started and mitral valve replacement resulted in clinical improvement. The postoperative course was complicated by haemodynamic instability, tachyarrhythmia, bilateral chylothorax, and respiratory insufficiency. At 4 months of life, a progressive discrepancy between OFC and other growth parameters with relative macrocephaly was observed. CT scan showed a mild distension of lateral ventricles with expansion of frontotemporoparietal subarachnoid spaces without intracranial hypertension. The electroencephalogram (EEG) registered poor global organization of electric activity and a slowed electrogenesis. The clinical examination revealed significant hypotonia, absence of subcutaneous adipose tissue, relative macrocephaly, neck skin redundancy, and absence of eyebrows and hair (Fig. 2.1c and d). To provide nutritional support, a percutaneous endoscopic gastrostomy (PEG) was placed. The evolving clinical phenotype suggested the diagnosis of Costello syndrome; mutation scanning of *HRAS* revealed no disease-related mutation. At age 6 months (Fig. 2.1e), recurrent episodes characterized by oculogyric crisis, nuchal rigidity, hypotonia, lockjaw, and transient loss of consciousness emerged. Subsequent EEGs confirmed the occurrence of electrophysiological anomalies (diffuse rapid dysrhythmia and central anomalies). Symptomatic partial epilepsy was diagnosed and an anticonvulsant therapy with phenobarbital and levetiracetam was started, with incomplete seizure control. The brain magnetic resonance imaging (MRI) showed

external benign hydrocephalus with enlarged subarachnoid spaces. At the last clinical evaluation at the age of 27 months, the significant growth delay with dystrophic appearance was confirmed, weight was 8260 g (< 3rd centile), height was 77 cm (< 3rd centile), OFC was 47.5 cm (10th–25th centile), as well as a severe developmental delay, mainly characterized by inability to sit unassisted and poor feeding. Hair was sparse, fine, and easily pulled from the scalp. Molecular analysis of *SHOC2* disclosed a de novo heterozygous c.4A > G substitution.

Patient 2 - The proband was the first child of Italian, healthy, and non consanguineous parents. He was born after an ICSI (intracytoplasmic sperm injection) pregnancy, performed for paternal infertility and complicated by pre-eclampsia. Prenatal history was characterized by fetal ultrasound anomalies: short femurs and intrauterine growth retardation (IUGR). He was delivered at 36 weeks gestation by caesarean. Birth growth parameters confirmed the poor intrauterine growth: weight was 2200 g (3rd–10^{th} centile), length 44 cm (3rd–10th centile), and OFC was 32.5 cm (50th centile). Apgar scores were 7 and 9 at 1 and 5 min, respectively. At birth, a cardiac murmur was detected, and echocardiogram showed a small patent foramen ovale (PFO) with a left to right shunt. Echoencephalography and renal US were normal. A preliminary neurologic evaluation, routinely performed in all preterm newborns, showed adequate spontaneous motor activity and normal neonatal reflexes. He was admitted at age 7 days for the association of congenital heart defect with growth failure. Relative macrocephaly, dystrophic appearance of subcutaneous adipose tissue, low set and posteriorly angulated ears, absence of eyebrows, and sparse, thin and easy pluckable hair were evident (Fig. 2.2).

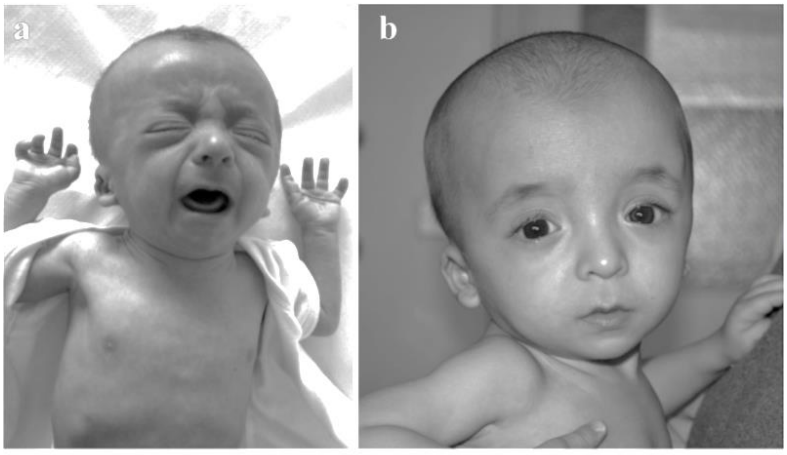

Fig. 2.2 Patient 2 at 7 days of life (a) and at 13 months (b)

The diagnosis of a RASopathy was considered, and mutation analysis of SHOC2 revealed a de novo heterozygous c.4A > G change. A second echocardiogram performed at age 7 months revealed the complete closure of the PFO with no other cardiac anomalies. At age 13 months (Fig. 2b), weight was 6.5 kg (< 3rd centile), height was 71 cm (< 3rd centile), OFC was 47 cm (50th centile), sparse eyebrows and hair were observed. At age 30 months, the growth failure with relative macrocephaly was confirmed: weight was 9.1 kg (<3rd centile), length 82 cm (<3rd centile), OFC 51 cm (75°centile). Gross motor developmental milestones have been achieved within normal range, a mild fine motor delay has been observed.

DISCUSSION

Rasopathies constitute an emerging group of clinically and genetically related disorders affecting development and growth; and having RAS signaling dysregulation as shared pathogenic mechanism [Tidyman and Rauen 2009; Zenker, 2011]. Within this family of developmental disorders, ten years ago, Mazzanti et al. [Mazzanti et al. 2003] recognized a distinctive phenotype characterized by short stature, macrocephaly, central nervous system anomalies, GH deficiency (GHD), and mild neurological developmental delay. Affected subjects had blonde, fine, sparse, and easily pluckable hair with prevalence of anagen hair at the trichogram. This condition was defined "Noonan-like syndrome with loose anagen hair". In 2009, Cordeddu et al. [Cordeddu et al. 2009] identified *SHOC2* as the disease gene underlying this condition. The phenotypic features of the original cohort including 25 affected individuals were relatively homogeneous, consisting of a NS-like facial appearance, growth failure frequently associated with GHD, mild neurocognitive impairment with hyperactive behavior, hair anomalies, and cardiac defects. Intriguingly, some of these subjects were reported to have features suggestive of Costello syndrome, especially in early infancy. More recently, a number of reports confirmed the distinctive phenotype associated with the c.4A > G mutation, but also provided evidence for a wider clinical variability characterizing this disorder [Komatsuzaki et al., 2010; Digilio et al., 2011; Lee et al., 2011; Capalbo et al., 2012; Hoban et al., 2012; Gripp et al., 2013; Şimşek-Kiper et al., 2013; Gargano et al., 2014; Zmolikova et al., 2014]. In this work, we reported two additional subjects heterozygous for the c.4A > G SHOC2 mutation, showing substantial differences in their phenotypic expression. While both patients presented with severe failure-to-thrive with characteristic dystrophic appearance and typical ectodermal phenotype, Patient 1 presented complex cardiomyopathy and a

critical CNS involvement with multidrug resistant epilepsy and severe neurodevelopmental delay, contrasting with the apparently normal neurological development of Patient 2. The peculiar ectodermal involvement observed in these patients, and their reduced growth appears to be the sole phenotypically invariable feature. Of note, occurrence of an atypical phenotype was recently reported in a subject with co-occurring mutations in SHOC2 and PTPN11 [Ekvall et al. 2011]. In that patient, the severe and complex phenotype was proposed to result from an additive effect, with the PTPN11 mutation acting as a modifier. This observation is in line with other studies reporting co-occurrence of RASopathy gene mutations in patients with variable NS or neurofibromatosis-NS phenotypes [Bertola et al. 2005; Nyström et al. 2009; Fahrner et al. 2012]. Based on these considerations, we cannot exclude that the complex and atypical phenotypes in NS/LAH might be associated with co-occurrence of additional RASopathy gene mutations or modifying loci acting on the RAS-MAPK cascade or functionally related signaling pathways. Targeted re-sequencing studies directed to genotype large and clinically well-characterized cohorts are required to test this hypothesis. Recently a novel mutation in SHOC2(c.519G>A; p.M173I) has been reported, leading a phenoype partially overlapping those occurring in NS [Hannig et al. 2014]. Phenotypic features of patients described by Hannig et al. were significantly different from those reported for patients with the c.4A>G; p.S2G SHOC2 mutation, presenting with craniofacial features suggestive of NS and sparse slow-growing hair. She does not have short stature, a cardiac disease, hyperpigmentation, ichthyosis or loose anagen hair and her developmental delays was relatively mild. This report confirm the hypothesis that SHOC2 mutations can cause a spectrum of phenotypes of varying severity.

Chapter 3.

Prognostic relevance of the prenatal features

This work has been published as

Baldassarre G, *Mussa A, Dotta A, Banaudi E, Forzano S, Marinosci A, Rossi C, Tartaglia M, Silengo M, Ferrero GB*. Prenatal features of Noonan syndrome: prevalence and prognostic value. Prenat Diagn. *2011 Oct;31(10):949-54.*

Baldassarre G*, Mussa A, Silengo M, Ferrero GB.* Comment on "prenatal diagnosis and prognosis in Noonan syndrome". *Prenat Diagn. 2013 Dec;33(13):1318-20*

BACKGROUND

Prenatal detection of syndromic and polymalformative patterns is a continuously evolving field and a challenge for gynecologists and clinical geneticists. Maternal serum tests, measurement of fetal nuchal translucency (NT) and morphologic ultrasound (US) are commonly used for the assessment of fetal health and screening for genetic and/or developmental defects. Before 2001, NS diagnosis was exclusively clinical. While a comprehensive scoring system has been developed to aid in diagnosis [van der Burgt *et al.* 1994], clinical assessment does not usually include prenatal features, although they are frequently observed in this syndrome. Several authors have suggested that, in absence of karyotype abnormalities, this syndrome should be considered in the differential diagnosis of foetuses presenting with increased NT, especially when cardiac defects, polyhydramnios, and/or multiple effusions are concomitantly observed [Benacerraf et al. 1989; Donnenfeld et al. 1991; Nisbet et al. 1999; Achiron et al. 2000; Hiippala et al. 2001; Schluter et al. 2005; Houweling et al. 2010]. The prevalence of prenatal anomalies in NS and their correlation with genotype and postnatal phenotype, however, has not been systematically investigated so far. The aim of the present study was to provide retrospective data on this matter.

PATIENTS AND METHODS

Patients - The cohort consisted of 47 patients, including 26 (55.3%) males and 21 (46%) females, with clinical features within the NS phenotypic spectrum. Mean age at diagnosis was 7.0 years (ranging from birth to 38 years). Clinical diagnosis was molecularly confirmed by analyzing the entire PTPN11, SOS1, KRAS, BRAF, RAF1

and SHOC2 coding sequences, as previously reported (Tartaglia et al. 2002; Carta et al. 2006; Pandit et al. 2007; Tartaglia et al. 2007; Cordeddu et al. 2009; Sarkozy et al. 2009). The majority of the subjects were heterozygous for mutations affecting the PTPN11 (N = 30, 68% of cases) or SOS1 (N = 8, 17%) gene. Most patients included in the study were sporadic cases inheriting a de novo germline mutation. Members from three families were also considered. In the first family, two sisters carried the common NS-causing c.188 A > G (Tyr63Cys) change (both parents refused the test). In the second family, two brothers inherited from the affected mother the c.854 T > C (Phe285Ile) change, that has been previously documented in NS. Finally, two sisters were found to inherit a c.1162 T > A (Leu261His) substitution from the father and the paternal grandfather, both exhibiting a mild phenotype which was only suggestive for NS. This sequence variant was not observed among 96 population-matched unaffected individuals and over 200 patients with a clinical diagnosis of NS with normal PTPN11 sequence, suggesting a causal link with the disorder. Karyotype analysis was normal in all patients, with the exception of a familial balanced translocation [45, XX der(13,14) (q10;q10)] observed in a female proband, in her father and in her healthy brother. Informed consent was obtained from all patients included in the study.

Prenatal and perinatal phenotype assessment - Prenatal and perinatal data were collected by careful analysis of medical records and anamnestic investigation, including: results of prenatal genetic screening tests analyses (maternal serum test and NT), occurrence of polyhydramnios and morphologic fetal US anomalies (hydrothorax, multiple effusions, malformations). In Italy, the first fetal US scan is usually performed at 11–13.6 weeks pregnancy as recommended by the Official Guidelines of the Italian Society of Gynecology and Obstetrics. An NT of 2.5 mm or

greater was considered abnormal. A second and third US are usually performed at 20–22 and 30–32 weeks of gestation.

Postnatal phenotype assessment - Postnatal phenotype was assessed as follows: (1) occurrence, age of clinical onset, and severity of CHD; (2) growth pattern; (3) neuropsychomotor development; (4) electroencephalography (EEG) anomalies, and/or epilepsy and (5) occurrence of haematological anomalies. To grade and standardize the clinical severity of the phenotype, a scoring system assigning one and three points respectively to the minor and major van der Burgt's criteria [van der Burgt, 1994] was developed, allowing to classify the phenotype as mild (score = 1–6), moderate (score = 7–12), or severe (score = 13–18). A second standardized scoring system was used to classify CHD, where score 0 refers to normal cardiac development, whereas score 4 refers to severe or lethal CHD. A similar method was applied to define neuropsychomotor development, ranging from normal achievement of developmental milestones and learning abilities (score 0) to significant speech delay associated with DD/ID (score 3). Short stature was graded as severe with height below the third percentile (score 2), moderate between the third and tenth percentile (score 1) or absent above the tenth percentile (score 0). Table 3.1 summarizes the scoring system proposed.

Table 3.1 - Scoring System

FEATURES	SCORING SYSTEM PROPOSED
Clinical phenotype based on van der Burgt's criteria 3 pts for each major feature, 1 pt for each minor feature*	1-6 = mild 7- 12 = moderate 13 – 18 = severe
Congenital Heart Disease (CHD)	0 = absent 1 = spontaneous resolution or not specific treatment required 2 = pharmacological treatment required 3 = surgical treatment required 4 = not resolutive surgery or heart transplantation or death
Neuropsychomotor development	0 = normal 1 = delayed achievement of developmental milestones 2 = learning disabilities 3 = learning disabilities and speech impairment
Growth pattern	0 = normal 1 = stature < 10° percentile 2 = stature < 3° percentile

Major feature include: typical facial dysmorphisms, PS, HCM and/or EEG anomalies, height < 3°centile, first degree relative with ddefinitive NS, pectus carinatuum/excavatum, mental retardation, cryptorchidism and lymphatic dysplasia **Minor feature include**: suggestive facial dysmorphisms, other cardiac defects, first degree relative with suggestive NS, broad thorax, none of mental retardation, cryptorchidism and lymphatic dysplasia

Statistics - Associations between prenatal features, genotype, and postnatal phenotype were explored among all the variables considered. Fisher's exact test or Chi square (χ^2) test was employed for nominal variables. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Our study group consisted of 47 patients with prenatal information, postnatal phenotype and molecular analysis available. Prenatal US had been performed in the entire patient group, whereas maternal serum test and measurement of NT were also determined in 22 subjects (46.8%). Among the prenatal findings, abnormal results at serum tests were reported in 8/22 cases (36%), increased NT in 9/22 (41%), polyhydramnios in 18/47 (38%), and abnormal sonographic fetal findings in 10/47 (21%). Any abnormal prenatal finding was present in 20/47 cases (43%). Table 3.2

summarizes the associations between prenatal features in the cohort and the postnatal scores of disease severity.

Table 3.2. Association between prenatal findings and postnatal scores

	Triple test results		NT results		Amniotic fluid volume		US fetal anomalies	
Score	Abnormal ($n = 8$)	Normal ($n = 14$)	Increased ($n = 9$)	Normal ($n = 13$)	Increased ($n = 18$)	Normal ($n = 29$)	Present ($n = 10$)	Absent ($n = 37$)
Clinical phenotype	$p = 0.979$		$p = 0.899$		$p = 0.158$		$p = 0.362$	
Mild	1	2	1	2	2	10	1	11
Moderate	5	9	6	9	11	15	6	20
Severe	2	3	2	2	5	4	3	6
CHD	$p = 0.924$		$p = 0.771$		$p = 0.344$		$p = 0.169$	
Score 0	1	1	1	1	2	4	0	6
Score 1	5	9	5	9	11	12	6	17
Score 2	0	1	0	1	0	2	0	2
Score 3	1	1	2	1	3	10	2	11
Score 4	1	2	1	1	2	1	2	1
Neuropsychomotor development	$p = 0.466$		$p = 0.293$		$p = 0.003$		$p = 0.009$	
Score 0	1	5	1	5	3	19	0	22
Score 1	6	6	7	5	11	6	7	10
Score 2	1	2	1	2	2	4	2	4
Score 3	0	1	0	1	2	0	1	1
Growth pattern	$p = 0.329$		$p = 0.530$		$p = 0.544$		$p = 0.584$	
Score 0	2	1	2	1	4	5	1	8
Score 1	1	5	2	5	3	9	2	10
Score 2	5	8	5	7	11	15	7	19

CHD, congenital heart defect; NT, nuchal translucency; US, ultrasound.

Overall, 50 CHD were documented in 41 patients (87.2% of the study cohort): 30 PS, 7 HCM, and 13 septal defects. However, only in four cases the CHD has been detected prenatally (9.7%): septal defects were observed in two patients, left superior vena cava in coronary sinus in the other two. On the basis of the scoring system referred to CHD, 23 subjects (56%) exhibited spontaneous resolution of the anomaly or did not required specific treatment (score 1), two individuals (4.8%) required pharmacological treatment (score 2), while in 13 cases (31.7%) a surgical treatment was necessary (score 3), and in three subjects (7.3%) surgery was not resolutive or the severity of the disease required heart transplantation or was the cause of death (score 4). No statistically significant association was observed between CHD and prenatal findings. Normal neuropsychomotor development — score 0 — was observed in 23 patients (49%). Delayed achievement of early neuromotor milestones with subsequent normal development (score 1) was observed in 15 subjects (31.9% of the entire cohort), DD/ID

(score 2) was observed in six individuals (12.7%), while in three patients (6.3%) DD/ID was associated with a severe speech delay (score 3). A significant statistical correlation was found between morphologic fetal US anomalies and DD/ID (scores 1–3) ($p < 0.001$), even though the statistical power of this association decreased when only significant neurodevelopmental anomalies (scores 1–2) were taken into consideration. No associations were observed between pathologic serum test and/or increased NT and neuropsychomotor score. Severe reduced growth occurred in 26 patients (55.3%), while moderate short stature was observed in 11 subjects (23.4%). No association was present between growth pattern and prenatal findings. Five patients (11%) presented hematological anomalies, with juvenile myelomonocytic leukemia (JMML) documented in four of them, and a myelodysplastic disorder in one, spontaneously resolved in all of them. Morphologic fetal US anomalies were more frequent among NS children with hematologic anomalies than without them (5/5 vs 5/42, $p < 0.001$).

DISCUSSION

Prenatal features and diagnosis of NS have been reported in several studies, but a clear correlation to the postnatal phenotype has not been investigated thoroughly. Several authors suggested that NS might be suspected when cystic hygroma, ascites or pleural effusion, hydrops fetalis, polyhydramnios, increased NT or CHD are observed [Benacerraf et al. 1989; Donnenfeld et al. 1991; Sharland et al. 1992, Nisbet et al. 1999; Achiron et al. 2000; Hiippala et al. 2001; Schluter et al. 2005; Houweling et al. 2010]. Sharland *et al.* reported polyhydramnios in the prenatal history of 33% of NS patients. The aetiology of this feature is still unclear, but it could be related to fetal

swallowing anomalies. In another report [Lee et al. 2009], it has been estimated that PTPN11 mutations are identifiable in 2 and 16% of fetuses presenting increased NT and cystic hygroma, respectively, confirming the poor specificity of these findings. As far as we are aware, this is the first report detailing the prenatal features of NS retrospectively by investigating a clinically well-characterized and relatively large cohort of patients with diagnosis confirmed molecularly. This report supports the view that a significant subset of subjects with NS present with a wide variable range of prenatal anomalies, the most frequent being polyhydramnios (38.3%), and increased NT (36.4%). Of note, these prenatal findings do not correlate with any investigated aspect of the postnatal phenotype, and the overall prognosis of the syndrome. Morphologic fetal US anomalies were detected in more than one fifth of subjects, with the most common being hydrothorax, observed in 10.6% of cases. Among these subjects, only 3 of the 10 patients with fetal morphologic US anomalies were characterized by a severe postnatal phenotype. Specifically, five of these subjects presented class 1 DD/ID with good prognosis, while five of them presented a more severe DD/ID (class 2, $N = 2$; class 3, $N = 3$). Interestingly, all the five patients presenting myelodysplastic syndrome (MDS/JMML) were characterized by a pathologic prenatal history of morphologic fetal US anomalies, with three of them presenting hydrothorax. The correlation between fetal multiple effusions and MDS/JMML is intriguing and deserves further analysis. Of note, most of the prenatal parameters investigated were unable to predict the postnatal outcome disclosing a very poor correlation with the prognosis. Anyway, prenatal US anomalies, mostly hydrothorax, were observed to correlate to an increased likelihood of MDS/JMML and DD/ID. The observed timing of diagnosis of CHD in the present cohort of patients, a key feature of NS, deserves specific comment. Specifically, only a minor fraction (8%)

of CHD observed in this cohort was diagnosed prenatally, whereas approximately half of them were clinically evident at birth. This study carries the limitation of the retrospective design, being unblinded and prone to recall bias. It should be also considered that to the accuracy of the study and to better evaluate eventual genotype–phenotype correlations, we only included patients with a positive molecular testing. As a consequence, the majority of subjects were PTPN11 mutation-positive patients. While no evidence of a significantly different distribution of prenatal anomalies among patients with mutations in different disease genes was apparent, specific correlations between prenatal and postnatal features in patients with mutations in genes weakly represented in the present cohort (i.e. SHOC2, KRAS and BRAF) cannot be ruled out. In conclusion, this study represents a retrospective investigation about prenatal phenotype in NS, and its correlation with the severity of the prognosis. Even though non-specific, prenatal anomalies are very frequent in NS pregnancies. Finally, although most of the prenatal features we described resulted not useful for the prediction of the phenotype evolution, some correlations between prenatal anomalies and postnatal phenotypic features are quite interesting and deserve further studies on larger cohorts to better define their meaning and to better understand their clinical implications. Afterwards we had the opportunity to extend our previous observations. We broadened our observations exploring the prenatal/postnatal phenotype correlations in a larger cohort of NS patients, including 74 cases with a clear-cut and molecularly confirmed diagnosis of NS and accurate collection of prenatal and postnatal data. Increased nuchal translucency was present in 42.5% (17/40), polyhydramnios in 33.7% (25/74), and fetal US anomalies (including hydrothorax or multiple effusions occurring in six cases, cardiac anomalies in five cases, intra-uterine growth restriction in three cases, nephro-urologic in two cases, and central nervous

system and intestinal malformations in single cases) were overall found in 32.4% (24/74) of cases. Lymphatic dysplasia was the most common fetal US anomaly observed (6/24), and significantly associated with the development of juvenile myelomonocytic leukemia/myelodysplastic syndrome (JMML/MDS) (4/6, $p < 0.001$) and epilepsy (3/9, $p = 0.021$); delayed neuropsychomotor development was observed in these six patients (four with delayed achievement of developmental milestones and two with learning disabilities). As expected, increased nuchal translucency and polyhydramnios were more common among patients with lymphatic dysplasia signs (4/5 vs. 7/26 of those without, $p = 0.046$ and 5/6 vs. 19/67, $p = 0.013$, respectively). No significant differences were detectable for the other prenatal findings, neither was possible to define genotype-phenotype correlations. Although there was not a clear-cut parallelism between prenatal findings and disease severity, it is interesting to note that the subset of patients with several (≥2) prenatal findings consistently display a more severe postnatal phenotype. The latter, empirically scored on Van der Burgt's criteria, paralleled the number of prenatal findings observed. On the other hand, no conclusion can be drawn for patients with no or isolated prenatal findings (<2), as they show an extreme variable phenotypic spectrum ranging from mild to severe forms (Figure 3.1).

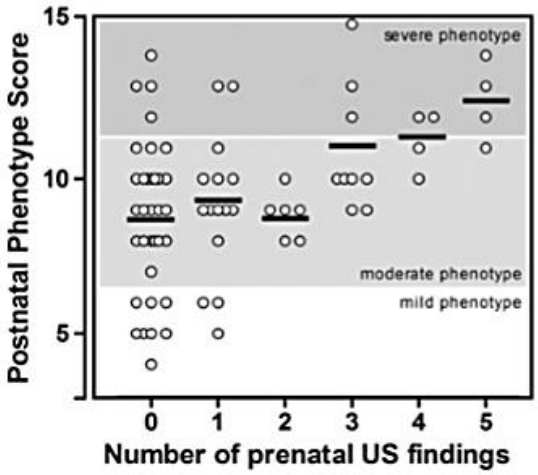

Fig. 3.14 Correlation between the number of prenatal findings (including fetal anomalies, increased nuchal translucency, polyhydramnios) and the postnatal phenotypic scores based on Van der Burgt's criteria (three points for each of the major features, one point for each of the minor ones) proposed. Patients with several prenatal findings (≥2) present high phenotypic scores, more likely within the severe phenotype spectrum. Bold horizontal lines represent the mean postnatal phenotype score in each group

Interestingly, a study of 2013 showed that fetuses with increased nuchal translucency and normal karyotype prenatally tested for the RAS-MAPK pathway gene panel were diagnosed with a RASopathy in more than 17% of cases [Croonen et al. 2013]. They more likely showed besides increased nuchal translucency additional US features as polyhydramnios, hydrops fetalis, renal anomalies, hydrothorax, cardiac anomalies, cystic hygroma, and ascites. This appears to be another clue indicating that the association of more than one prenatal anomaly appears to be a promising indicator of NS pregnancies. In conclusion, we have observed abnormal prenatal findings in approximately half of the pregnancies of our NS cohort, mostly nonspecific and unreliable for the prediction of NS postnatal phenotype. However, lymphatic dysplasia is the unique prenatal finding displaying association with juvenile myelomonocytic leukemia/myelodysplastic syndrome and central nervous system involvement (developmental delay and epilepsy) of the postnatal presentation of NS. Application of next-generation sequencing technologies to prenatal invasive and/or noninvasive

diagnosis will allow a growing numbers of molecularly based diagnoses of Mendelian conditions for which the prognosis definition based on prenatal features will be a key point of the clinical approach. In this respect, NS represents a paradigmatic condition for which clinical research based on molecularly well-defined cohorts, thorough collection of prenatal features and deep analysis of postnatal phenotype is clearly needed.

Chapter 4.

Functional Hematopoietic profile

This work has been published as

Timeus F, Crescenzio N, ***Baldassarre G****, Doria A, Vallero S, Foglia L, Pagliano S, Rossi C, Silengo MC, Ramenghi U, Fagioli F, Cordero di Montezemolo L, Ferrero GB.* Functional evaluation of circulating hematopoietic progenitors in Noonan syndrome. *Oncol Rep. 2013 Aug;30(2):553-9.*

BACKGROUND

The association between NS and myeloproliferative disorders, in particular JMML, has been deeply investigated in several studies [Choong et al. 1999, Kratz et al. 2005, Bastida et al. 2011, Strullu et al. 2014]. Both NS and JMML are characterized by hyperactivation of the RAS/MAPK signaling pathway. NS is associated with germline PTPN11 mutations in ~50% of the patients, while somatic PTPN11 mutations are found in 35% of children with JMML. The PTPN11 mutational spectrum has been shown to be different in JMML, NS/MPD and NS without any hematological abnormalities [Kratz et al. 2005]. A myeloproliferative disorder (NS/MPD) can occasionally be diagnosed in infants with NS. The clinical course of NS/MPD is usually benign with spontaneous remission. However, various cases with an aggressive course resembling juvenile myelomonocytic leukemia (JMML) have been described [Bader-Meunier et al. 1997, Fukuda et al. 1997, Choong et al. 1999, Bastida et al. 2011]. JMML is a rare clonal myelodysplastic-myeloproliferative disorder typical of infancy and early childhood, characterized by spontaneous in vitro proliferation of bone marrow and peripheral blood hematopoietic progenitors in the absence of exogenous growth factors, due to selective hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) [Emanuel et al. 1991]. Hepatosplenomegaly, lymphadenopathy, anemia, thrombocytopenia, and fever, variably associated with symptoms of non-hematopoietic organ infiltration, are common clinical findings in JMML. The fulfillment of the following laboratory criteria is required for JMML diagnosis: an absolute monocyte count >1,000/μl, <20% bone marrow blasts and the absence of t (9;22) or BCR/ABL rearrangement. Apart from such mandatory criteria, JMML patients may present with a high white blood cell count (>10,000/μl), immature myeloid precursors on a peripheral smear and increased

fetal hemoglobin (HbF) for age. Monosomy 7 is quite frequently noted [Hasle et al. 2003]. It has been previously demonstrated that flow cytometric evaluation of the absolute count of peripheral blood (PB) CD34+ cells and the apoptotic rate is a simple and repeatable technique, useful for early detection of clonal evolution in acquired aplastic anemia. In this study, we performed a functional evaluation of the circulating hematopoietic progenitors in a series of NS patients. Clonogenic tests in the absence or in the presence of increasing concentrations of GM-CSF and three-color flow cytometric analysis for CD45, CD34, and Annexin V were performed using the PB of 27 patients with NS and 5 patients with JMML. The different functional patterns were compared to identify a possible NS subgroup worthy of stringent hematological follow-up for an increased risk of MPD development.

PATIENTS AND METHODS

Twenty-seven patients with a clinical and molecular diagnosis of NS were enrolled in the study. Three patients had a myeloproliferative disorder (NS/MPD), with monocytosis, atypical monocytoid cells, myelodysplastic features and granulocyte precursors in PB, thrombocytopenia and hepato-splenomegaly. Five patients with a diagnosis of JMML, fulfilling the EWOG-MDS criteria [Hasle et al. 2003] were also included. PB samples were collected in EDTA at diagnosis. Further blood samples were collected and analyzed in NS patients when hematological anomalies (e.g. anemia, thrombocytopenia, leukocytosis, splenomegaly, lymphadenopathy) and/or alterations of the functional pattern of circulating hematopoietic progenitors were observed. NS/MPD and JMML patients were evaluated at various stages during follow-up and treatment. Ethics committee approval and informed consent of the parents of the patients were obtained.

Molecular analysis - Genomic DNA was isolated from 200 μl of PB by the QIAamp DNA Blood Mini kit (Qiagen, Germantown, MD, USA). A molecular analysis of PTPN11, KRAS, SOS1, RAF1, BRAF, SHOC2, NRAS and CBL was performed as previously described.

Absolute count of CD34+ cells and the apoptotic index - Flow cytometric analysis was performed within 2 hours after venipuncture. The absolute count of CD34+ cells and the apoptotic rate were evaluated by a three-color fluorescence for CD45, CD34 and Annexin V as follows. A total of 5×105 nucleated cells were incubated for 20 min at 4°C with anti-CD34 PE (8G12; Becton-Dickinson, San José, CA, USA) and anti-CD45 PerCP (2D1; BD Biosciences, Franklin Lakes, NJ, USA). After incubation and red cell lysis by ammonium chloride, the samples were washed in cold phosphate-buffered saline (PBS) and incubated with Annexin V-fluorescein isothiocyanate (FITC) (Apoptosis Detection kit; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The cells were then analyzed in a Coulter Epics XL2 (IL, Bedford, MA, USA) cytometer equipped with an argon laser. CD34+ cells were identified by a sequential gating strategy according to the ISHAGE protocol (26). Absolute CD34 counts were assessed by a two-platform method with a Sysmex K4500 counter (Sysmex Corporation, Kobe, Japan). At least 100 CD34+ cells were evaluated in each experiment.

Cell cultures - Low-density mononuclear cells (2×105) obtained from the patient PB by density centrifugation over Ficoll-Hypaque gradient were plated in multi well plates in 250 μl Iscove's modified Dulbecco's medium (IMDM) containing 30% fetal calf serum (FCS) (both from Sigma-Aldrich, St. Louis, MO, USA), 0.3% noble agar and 100 U/ml rhIL-3 and decreasing concentrations (20, 10, 5, 1, 0.1 ng/ml) of rhGM-CSF (both from Invitrogen Life Technologies, Carlsbad, CA, USA). After 14 days, single aggregates of >40 cells were scored as CFU-GMs. GM-CFU assay was also performed without GM-CSF stimulation. GM-CFU assay in the same culture conditions was also performed in 21 pediatric controls (median age, 9.0; range, 1–18 years).

Statistical analysis - The patients were divided into 3 groups: NS, NS/MPD and JMML. Historical controls (n=68) from our laboratory were utilized for the absolute count of CD34+ in PB and the apoptotic rate. For the absolute count of CD34+ cells and apoptotic rate, the values for each patient were compared with the mean control value adjusted for age, as previously published [Timeus et al. 2005]. The cell culture data and the absolute count of CD34+ cells and the apoptotic rate results were analyzed using the non-parametric Kruskal-Wallis test. Pairwise comparisons for the disease groups were performed for each of the GM-CSF concentration utilized in the cell cultures, as well as for the 4 variables analyzed in the test (absolute CD34+, percentage of CD34+/Annexin V+, absolute CD34+ to the mean age group value ratio, percentage of CD34+/Annexin V+ to the mean age group value ratio). Fisher's exact probability test was utilized for correlations between the groups.

RESULTS

Mutational spectrum - The mutational spectrum of our series of NS, NS/MPD and JMML patients is shown in Table 4.1.

Table 4.1. Mutational spectrum, WBC, PLT and monocyte counts, CD34+ absolute count and apoptotic rate, CFU-GM from peripheral blood in our NS, NS/MPD and JMML patients.

Patient	Mutated gene	Mutation	Hypersensitivity to GM-CSF	Unstimulated colony growth	Annexin V+/CD34+ (%)		CD34 (/μl)		WBC (10^3/μl)	Monocytes (10^3/μl)	PLT (10^3/μl)
NS1	PTPN11	Gln79Arg	No	No	8.5	L	3.6	N	11.9	0.740	284
NS2	PTPN11	Asn58His	No	No	26.1	H	7	N	11.6	0.400	262
NS3	PTPN11	Tyr63Cys	No	No	16.1	N	2.5	L	8.3	0.300	428
NS4	PTPN11	Gly503Arg	No	No	2.56	L	3.42	L	17.1	1.720	331
NS5	PTPN11	Gly503Glu	No	No	7.6	L	3.6	N	7.3	0.900	249
NS6	PTPN11	Asn308Ser	No	No	12	L	6.2	H	10.3	0.690	297
NS7	PTPN11	Leu261His	No	No	10	L	2.9	N	5.8	0.470	416
NS8	PTPN11	Leu261His	No	No	0	L	4.8	N	12.0	0.770	376
NS9	PTPN11	Leu261His	No	No	10	L	17.52	H	7.3	0.600	277
NS10	PTPN11	Phe285Ile	No	No	7.9	L	3.8	N	7.7	0.410	218
NS11	PTPN11	Glu76Asp	Yes	Yes	1.0	L	12	H	12.0	1.140	300
NS12	PTPN11	Glu139Asp	No	No	17.8	L	2.2	L	7.3	0.510	198
NS13	PTPN11	Asp61Asn	Yes	No	4.1	L	13.8	H	15.3	0.840	212
NS14	SOS	Ile252Thr	No	No	27.7	H	2.6	L	6.4	0.450	259
NS15	SOS	Thr266Lys	No	No	8.8	L	16.2	H	12.5	0.620	291
NS16	SOS	Arg552Gly	No	No	18.2	L	1.3	L	4.3	0.340	259
NS17	SOS	Glu433Lys	No	No	0	L	7.75	N	15.5	1.420	422
NS18	RAF1	Ser257Leu	No	No	8	L	2.5	L	6.4	0.540	253
NS19	SOS	Met269Thr	Yes	No	4.4	L	5.2	N	8.7	1.100	182
NS20	n.d.	n.d.	No	No	11.2	N	4.5	L	12.2	1.110	184
NS21	KRAS	Gln22Arg	No	No	15.7	L	5.6	N	9.44	0.630	551
NS22	BRAF	Leu597Val	No	No	0.09	L	7.1	H	7.9	1.600	397
NS23	RAF1	Pro261Ser	No	No	25	H	5	N	12.6	1.450	384
NS24	SHOC2	Ser2Gly	No	No	6.8	L	8.5	N	17.0	0.700	482
NS/MPD1	PTPN11	Phe285Ser	Yes	Yes	2.4	L	58.0	H	14.5	2.500	399
NS/MPD2	PTPN11	Asp61Asn	Yes	Yes	0.2	L	1,374	H	43.5	5.520	54
NS/MPD3	PTPN11	Asp61Asn	Yes	No	1.6	L	205.7	H	20.2	2.550	167
JMML1	n.d.	n.d.	Yes	Yes	2.1	L	193.6	H	9.3	1.900	55
JMML2	PTPN11	Glu76Gly	Yes	Yes	12.1	N	44	H	8.8	1.380	31
JMML3	PTPN11	Gly503Val	Yes	No	7.2	L	49.6	H	3.1	1.070	45
JMML4	NF1	n.d.	Yes	Yes	0.4	L	109.8	H	11.2	1.430	15
JMML5	n.m.	n.d.	Yes	Yes	0.4	L	232	H	36.2	7.610	71

Monocyte counts - All JMML patients showed monocytosis >1,000/μl. Ten out of the 27 NS patients showed monocytosis >1,000/μl, which included the 3 NS/MPD patients (Tab. 4.2)

Table 4.2. Circulating monocytes, peripheral blood CD34+ cells, their apoptotic rate and CFU-GMs in a series of NS, NS/MPD and JMML patients

Clinical parameters	Controls	NS	NS/MPD	JMML
Monocytes (/μl)	600 (200-900)	695 (300-1,720)	2,550 (2,500-5,520)	1,600 (1,070-7,600)
$CD34^+$ (/μl)	5.2 (1.8-23.1)	4.9 (1.3-17.5)	205.7 (58-1,374)	109.8 (44-232)
Annexin $V^+/CD34^+$ (%)	17.6 (2.8-49.6)	8.6 (0.0-27.7)	1.4 (0.2-2.4)	2.1 (0.4-12.1)
GM-CSF (20 ng/ml)	9 (0-26)	4 (0-34)	36 (0-84)	46 (18-258)
GM-CSF (10 ng/ml)	5 (0-18)	2 (0-30)	38 (0-76)	40 (10-244)
GM-CSF (5 ng/ml)	3 (0-18)	1 (0-24)	38 (0-50)	42 (6-262)
GM-CSF (1 ng/ml)	1 (0-16)	1 (0-23)	34 (0-62)	32 (6-264)
GM-CSF (0.1 ng/ml)	0 (0-14)	0 (0-8)	26 (0-36)	30 (2-240)
Unstimulated	0 (0-0)	0 (0-4)	4 (0-32)	18 (0-84)

NS, Noonan syndrome patients (n=24); NS/MPD, Noonan syndrome patients with myeloproliferative evolution (n=3); JMML, myelomonocytic leukemia patients (n=5). CFU-GMs from $2x10^5$ circulating mononuclear cells were cultured in the presence of decreasing GM-CSF concentrations and without GM-CSF (unstimulated). Data are provided as mean value (range).

Platelet counts - All JMML patients showed thrombocytopenia as well as one out of the 3 NS/MPD patients. In the other NS patients, the platelet counts were in the normal range, without a correlation with monocyte counts (R=0.016) (Tab 4.1).

Absolute CD34+ cell count and apoptotic rate - The PB absolute CD34+ cell counts and apoptotic rates in the different groups of patients are shown in tables 4.1 and 4.2. In JMML and NS/MPD patients, we observed high levels of circulating CD34+ cells with a low apoptotic rate. In NS patients, CD34+ cell counts were normal, whereas their apoptotic rate was significantly lower than that in the controls ($p<0.01$). Concerning the absolute CD34+cell count, statistically significant differences were noted among the NS and JMML ($p=0.001$), NS and NS/MPD ($p<0.05$), controls and JMML ($p<0.01$), and controls and NS/MPD patients ($p<0.05$). In contrast, no differences in the absolute CD34+ cell count were observed between the controls and NS or between the NS/MPD and JMML patients. Normalizing the absolute CD34+cell count for age (absolute CD34+ to mean age group value ratio), the results from the pairwise comparison did not change. A pairwise comparison of the Annexin V+ percentage showed a statistically significant decrease in the apoptotic rate in each disease group when compared with the controls: NS ($p<0.01$), NS/MPD ($p<0.05$), JMML ($p<0.01$); whereas no significant difference was observed between NS/MPD and JMML, JMML and NS, and NS/MPD and NS patients. When the percentage of Annexin V+ cells was normalized for age (percentage of Annexin V+/CD34+ cells to mean age group value ratio), the results for the pairwise comparison did not change.

Cell cultures - In JMML patients, the clonogenic assays from PB showed hypersensitivity to GM-CSF and spontaneous CFU-GM growth (except in one patient previously treated with chemotherapy at another center who did not show spontaneous CFU-GM growth) (Tab. 4.1). In 3 out of 3 NS/MPD patients, we observed hypersensitivity to GM-CSF and in 2 out of 3 spontaneous CFU-GM growth was noted. In two NS patients (NS13 and NS19) we observed hypersensitivity to GM-CSF, and no spontaneous CFU-GM growth. One NS patient (NS11) showed hypersensitivity to GM-CSF and spontaneous CFU-GM growth. This patient had a favorable clinical outcome, and the clonogenic assays performed 6 months later showed normal results. We observed a significant difference in the distribution and in the median values of CFU-GM among the 3 groups (JMML, NS, NS/MPD) (Tab. 4.2). Pairwise comparisons of GM-CSF-stimulated clonogenic assays showed that at different GM-CSF concentrations, JMML patients were significantly more responsive to GM-CSF than both the controls and NS patients. Concerning unstimulated cultures, a significant growth advantage was observed also in NS/MPD when compared with the controls and NS patients. Specifically, clonogenic assays without GM-CSF were able to distinguish between NS and NS/MPD patients. Fisher's exact probability test showed a significant correlation between the groups in the unstimulated colony growth tests (0% in controls, 4.2% in NS, 80% in JMML, 66.7% in NS/MPD, $p<0.001$)

Follow-up of NS and NS/MPD patients – Six NS patients showed isolated monocytosis (monocytes > 1,000/μl), 2 NS patients showed isolated hyper-responses to GM-CSF, 5 NS patients showed an isolated increase in circulating CD34+ cells. For all of these patients a 12-month follow-up showed no alterations in clinical findings. Patients NS11 and NS/MPD1 showed monocytosis, a hyper-response to GM-CSF, CFU-GM growth without GM-CSF stimulation, high circulating CD34+ counts with a low apoptotic rate. In these two NS patients a clinical and laboratory follow-up was performed. Patient NS11, carrying the Glu76Asp PTPN11 mutation, was phenotypically characterized by polyhydramnios in the prenatal history, typical facial dysmorphisms, pulmonic stenosis, bilateral cryptorchidism and normal neuropsychomotor development. His clinical follow-up in the following months was normal, and a laboratory hematologic evaluation performed 12 months later showed normal response to GM-CSF, no spontaneous colony growth and normal CD34+ cell and monocyte counts. Patient NS/MPD1 was first evaluated at the age of 2 months while she was in the cardiac surgery unit due to obstructive hypertrophic cardiomyopathy. A clinical diagnosis of NS was confirmed by the molecular analysis of the PTPN11 gene, that revealed the Phe285Ser mutation. A preliminary hematological evaluation evidenced only mild hepatomegaly and monocytosis. Clonogenic assays and flow cytometry showed hypersensitivity to GM-CSF, spontaneous CFU-GM growth, increased circulating CD34+ cells with a low apoptotic rate. At the age of 12 months, the patient showed splenomegaly, thrombocytopenia and monocytosis, with the presence in the peripheral blood smear of 10% of atypical monocytoid cells, moderate myelodysplastic features and granulocyte precursors. Bone marrow aspirate showed mild myelodysplasia and the presence of 15% of atypical monocytoid elements. A polymerase chain reaction (PCR) for bcr/abl

rearrangement was negative. The HUMARA assay performed on peripheral blood populations was normal, excluding a frank JMML evolution and suggesting a polyclonal myeloproliferative disorder, described in NS [Bader-Meunier et al. 1997]. Hence, a diagnosis of NS/MPD was made. Thrombocytopenia and splenomegaly persisted over the following months, and the clinical course worsened, with a progressive development of lymphatic dysplasia with thoracic duct ectasia, progressive severe respiratory insufficiency, pleural effusion and *exitus* at the age of 20 months for acute cardiopulmonary failure. Patients NS/MPD2 and NS/MPD3 were diagnosed in the first month of life presenting a myeloproliferative disorder. Table 4.3 shows the clinical and laboratory follow-up of the NS/MPD patients.

Tab. 4.3 Functional follow-up of 3 NS/MPD patients with myeloproliferative evolution.

	NS/MPD1		NS/MPD2		NS/MPD3	
Parameters	T_0	T_1	T_0	T_1	T_0	T_1
Age at T_0	2 months		10 days		1 month	
WBC (/μl)	14,500	8,200	43,500	8,480	20,200	16,000
Monocytes (/μl)	2,500	1,400	5,520	620	2,550	1,280
$CD34^+$ (/μl)	58	150	1,374	13	205	45
Annexin V^+ (%)	2.4	7.5	0.2	0.8	1.6	3.0
Hyper-response to GM-CSF	+++	++	+++	No	No	+++
Spontaneous CFU growth	++	++	+	No	No	No
Circulating dysplastic monocytes with myeloid dysplasia	No	+++	++	No	+	No
Hepatosplenomegaly	No	++	No	No	++	No
Thrombocytopenia	No	+++	++	No	No	No

NS/MPD, Noonan syndrome patients with myeloproliferative evolution. T_0, first observation; T_1, observation at 12 months.

DISCUSSION

Genetic diseases associated with a high tumor risk are models for the study of carcinogenesis. A close correlation is often observed between such genetic conditions and specific acquired neoplastic diseases. NS and JMML were shown to be strictly correlated to each other. Indeed, the same signal transduction pathway (RAS/MAPK)

is hyperactivated both conditions, with an involvement of the same genes. Moreover, NS patients presented an increased risk of developing JMML or, more frequently, a transient myeloproliferative disorder associated with Noonan syndrome (NS/MPD). Strictly correlated to the hyperactivation of the RAS/MAPK pathway is the hypersensitivity to GM-CSF observed in JMML [Emanuel et al. 1991]. In this study, we conducted a molecular study on a cohort of NS and JMML patients with a functional evaluation of their circulating hematopoietic progenitors, and correlated the results with the clinical-hematological course. In particular, our aim was to evaluate the circulating CD34+ cell count and apoptotic rate and to relate such findings with in vitro colony growth (in the absence and with increasing concentrations of GM-CSF), hematological features and the clinical history of each patient. The analyses were performed on PB, even though the biological variability of hematopoietic progenitor counts and clonogenic assays in PB is higher than in bone marrow. A bone marrow aspirate would have been unethical in NS patients without any sign of a hematological disease. Even though a constitutional GM-CSF hypersensitivity has been suggested in NS [Bastida et al. 2011, Lavin et al.2008], we observed hypersensitivity to GM-CSF in only 6 of the 27 NS patients. One of them developed a myeloproliferative disorder 12 months later (NS/MPD1) and two patients (NS/MPD2 and NS/MPD3) had a transient myeloproliferative disorder at the time of the study. In patient NS/MPD3, a hyper-response to GM-CSF was observed during the follow-up, but not at the diagnosis. The other three NS patients with GM-CSF hypersensitivity had normal hematological profiles. These data suggest that the response to GM-CSF is variable in NS patients. PTPN11 mutations in JMML affect different amino acids residues from those involved in NS. Somatic JMML-associated mutations are predicted to result in a stronger SHP-2 gain of function than germ-line mutations described in NS, and the

leukemic transformation in NS seems related to cooperating molecular lesions. In our NS series, one patient (NS5) carried the Gly503Glu PTPN11 mutation, also described in JMML. Interestingly, this patient showed a normal hematological profile without hypersensitivity to GM-CSF, but his mother, who presented with a short stature and typical facial appearance, as revealed by anamnestic data and family photographs, died due to non-Hodgkin lymphoma. PB CD34+ cell counts in the majority of our NS patients were normal, but the apoptotic CD34+ cell rate was significantly lower than in controls, as in JMML and NS/MPD. Previous studies have pointed to an increased proliferative activity of hematopoietic progenitors in NS. Our results allow us to identify NS as a disease with a lower-than-normal apoptotic activity of circulating hematopoietic progenitors. This increase in the survival of hematopoietic progenitors appears to be a hallmark of NS patients. The present data denote the complex hematopoietic functional profile of NS patients. It also suggests that the absolute CD34+ count, the apoptotic rate and the CFU-GM assay performed on PB could be a non-invasive and repeatable method to identify NS patients with a higher risk of myeloproliferative evolution. For these subjects an intensified hematological follow-up program could be justified.

Chapter 5.

Bone and mineral assessment

This work has been published as

***Baldassarre G**, Mussa A, Carli D, Molinatto C, Ferrero GB.* Constitutional bone impairment in Noonan syndrome. *Am J Med Genet A. 2017 Mar;173(3):692-698*

BACKGROUND

Short stature and skeletal dysmorphisms are constant phenotypic features of NS and Rasopathies and several studies hypothesized that the RAS pathway may regulate bone metabolism and homeostasis [Yu et al. 2005; Stevenson et al. 2011; Stevenson and Yang. 2011; Choudhry et al. 2012; Petramala et al. 2012; Rhodes et al. 2015]. However, except NF1, which has been largely explored both from a clinical and biochemical viewpoint, data on skeletal mineralization, bone metabolism and fracture rate are scanty in the other Rasopathies. In this work, we investigated the bone quality and the metabolic bone profiling in a group of patients with a molecularly confirmed diagnosis of NS.

PATIENTS AND METHODS

Patients - Thirty-five patients, admitted to our department from January 2006 to December 2015, were enrolled in the study. The cohort included 20 males (55.6%) and 15 females (44.5%) aged 1.0–17.8 years (mean 6.4 ± 4.5, median 4.9 years) with a molecular confirmed diagnosis of NS, including one with NS/ loose anagen hair (LAH). Mutational analysis of the Ras/MAPK causative genes was performed by analyzing the entire PTPN11, SOS1, KRAS, BRAF, RAF1, RIT1, and SHOC2 coding sequences, as previously reported [Tartaglia et al. 2001, 2007; Carta et al. 2006; Pandit et al. 2007; Cordeddu et al. 2009; Sarkozy et al. 2009; Aoki et al. 2013]. Twenty-five subjects (25/35—71.4%) harbored mutations in the *PTPN11* gene, 5/35 (14.2%) in the *SOS1* gene, 2/35 (5.7%) in the *RAF1* gene, 1/35 (2.8%) in the *BRAF1*, *KRAS*, and *SHOC2* genes, respectively. Informed written consent was obtained from all patients.

Clinical, laboratory and ultrasound evaluation - Each patient was submitted to clinical examination, X-Ray of the left hand for bone age evaluation, laboratory assays, and quantitative ultrasound (QUS). Phenotype was evaluated considering growth pattern, occurrence and severity of CHD, neuropsychomotor development, and/or hematological anomalies. Weight, length, and standing height was assessed by a standard clinical balance, an infantometer, and a Harpenden stadiometer (Holtain Limited, Crymych, Dyfed, UK). Values were compared with standardized growth curves for the Italian population [Cacciari et al. 2006] and expressed as a standard deviation score (SDS). Values below −2 SDS were considered pathologic, as for other bone appraisal techniques [Crabtree et al. 2014]. Body mass index (BMI) was calculated using the standard formula weight/height^2 (Kg/m^2) and reported as SDS. The pubertal stage was assessed according to Tanner criteria by physical examination of the testicular volume in males, breast development in females, and pubic hairs in both. Skeletal maturity was evaluated by left hand X-Ray and calculated according to Tanner and Whitehouse maturity score [Tanner et al. 1988]. Markers of bone metabolism including serum calcium, phosphate, 25-hydroxy vitamin D, 1,25-diyhdroxy vitamin D, parathyroid hormone, alkaline phosphatases, and IGF1 were obtained from peripheral blood samples. Dosage of urinary creatinine, phosphate, calcium excretion, and calcium/creatinine ratio was also performed. All patients underwent phalangeal QUS using DBM Sonic Bone Profiler 1200 (Igea, Carpi, Italy). This device consists of an electronic caliper with emitter and receiver probes recording ultrasound modifications through the distal metaphysis of the proximal phalanges of fingers II–V of the dominant hand. All measurements were performed by the same trained operator. The caliper was placed on the metaphysis and scanned around the finger, recording amplitude-dependent speed of sound (AD-SoS, m/s), and bone

transmission time (BTT, μs), two parameters correlating with bone properties. Technical details concerning the measurement have been already reported elsewhere [Mussa et al. 2012]. Absolute values of AD-SoS and BTT were standardized according to sex and expressed as SDS corrected for age, height, bone age, pubertal stage, and BMI [Baroncelli et al. 2006].

Statistical analysis - Statistics were conducted using Student t-test for continuous variables and Fisher exact test for discrete ones. Correlations were explored by Pearson method for parametric variables.

RESULTS

Age, height, bone age, puberty, and BMI-corrected AD-SoS SDS were -0.34 ± 1.93, 0.32 ± 1.88, -0.28 ± 2.03, -0.90 ± 1.46, and -0.89 ± 1.65, respectively; puberty-corrected and BMI-corrected SDS were significantly below normal ($P < 0.001$ and $P = 0.003$, respectively). All BTT SDS values were below the normal range: age-corrected (-1.40 ± 1.44, $P < 0.001$), height-corrected (-0.56 ± 1.15, $P = 0.007$), bone age-corrected (-1.27 ± 1.49, $P < 0.001$), puberty-corrected (-1.75 ± 1.29, $P < 0.001$), and BMI-corrected (-1.41 ± 1.27, $P < 0.001$) (Fig. 5.1).

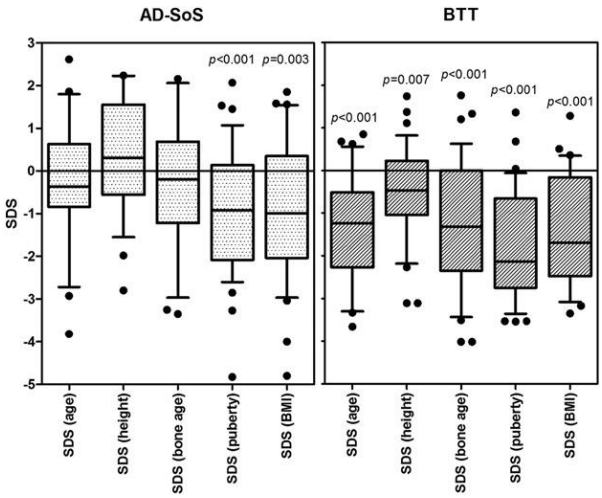

Fig. 5.1 Standard deviation score (SDS) of amplitude-dependent speed of sound (AD-SoS, left panel) and bone transmission time (BTT, right panel) corrected for age, height, bone age, pubertal stage, and body mass index (BMI). Boxes represent 25th–50th percentile, whiskers 10th–90th percentiles. Outliers are displayed as filled circles. P values refer to the comparison with normal SDS (zero value, displayed as a horizontal line) by student t test.

Based on BTT, 9/35 patients were below the −2 SDS threshold according to their age, 5/35 after height-correction, 10/35 after bone age-correction, 19/35 after pubertal stage correction, and 14/35 after BMI correction. According to AD-SoS, 4/35 patients were below-2 SDS following age correction, 2/35 following height correction, 4/35 following bone age correction, 9/35 following puberty and BMI correction respectively. Age, height, bone age, and BMI corrected BTT SDS were lower in males than in females; no differences were found between sexes in the other QUS, clinical, and biochemical parameters investigated. No differences were detectable based on genotype (PTPN11 vs. SOS1 and PTPN11 vs. other molecular defects). None but one of the patients had abnormal laboratory test (Table 5.1).

Tab. 5.1 Summary of the Clinical and Biochemical Variables Investigated

	Laboratory range	Mean ± SD	Median	Cohort range
Age	n.a.	6.40 ± 4.50	4.90	10.00–17.80
Height	n.a.	107.80 ± 23.3	107.10	71.00–170.00
Height SDS	n.a.	−1.89 ± 1.14	−1.70	−4.70 to 0.51
Pubertal stage				
B (breast)	n.a.	1.00 ± 0.96	1.00	1.00–5.00
G (genitalia)	n.a.	2.00 ± 1.34	1.00	1.00–5.00
Pubertal stage SDS	n.a.	−0.86 ± 1.45	−0.85	−4.80 to 2.07
Weight	n.a.	19.97 ± 11.75	16.75	7.20–71.00
Weight SDS	n.a.	0.14 ± 1.82	−0.02	−5.80 to 4.74
Body mass index (BMI)	18–25	16.16 ± 2.25	15.91	11.80–24.57
BMI SDS	n.a.	−0.84 ± 1.59	−0.75	−4.80 to 1.89
PTH (pg/ml)	7.0–83.0	55.3 ± 22.9	49.2	19.80–101.00[a]
25 OH vitamin D (ng/ml)	9.0–37.6	19.2 ± 11.9	17.1	2.60–33.50
1,25 OH vitamin D (pg/ml)	19.9–67.0	57.8 ± 24.0	58.8	4.60[a]–65.2
Serum calcium (mEq/L)	4.5–5.6	4.9 ± 0.8	4.9	4.2–5.6
Serum phophorus (mg/dl)	4.0–6.5	4.8 ± 0.5	4.8	4.70–6.24
ALP (U/L)	120.0–350.0	199.9 ± 116.2	174.0	1277.00–282.00
Urinary calcium/creatinine ratio	0.00–0.42	0.13 ± 0.10	0.11	0.01–0.39
IGF1 (ng/ml)	50.0–426.0	97.1 ± 75.9	69.3	25.00–359.00

Correlations were searched among all the continuous variables studied. AD-SoS SDS was negatively correlated with patients' BMI ($r = -0.36$, $P = 0.033$), BTT SDS showed a positive correlation with height SDS ($r = 0.17$, $P = 0.004$). PTH was negatively correlated with 1,25-(OH)2-vitamin-D ($r = -0.39$, $P = 0.034$), serum calcium ($r = -0.61$, $P < 0.001$), and phosphate ($r = -0.39$, $P = 0.030$), urinary calcium/creatinine ratio ($r = -0.49$, $P = 0.008$), and positively with alkaline phosphatase ($r = 0.9$, $P < 0.001$). Serum calcium correlated with 25-OH-vitamin-D ($r = 0.43$, $P = 0.016$), 1,25-(OH)2-vitamin-D ($r = 0.40$, $P = 0.024$), phosphate ($r = 0.54$, $P < 0.001$), and alkaline phosphatase ($r = -0.58$, $P < 0.001$). In our cohort, 3 patients reported previous fractures (8.6%): all occurred at long tubular bones (radius, clavicle, phalanges), following a high-energy traumatism, and healed normally. All fractures happened more than one years before QUS measurement.

DISCUSSION

In the last years, numerous studies explored the effects of the Ras/MAPK pathway deregulation on the skeletal development and bone metabolism [Yu et al. 2005; Stevenson et al. 2011; Stevenson and Yang, 2011; Choudhry et al. 2012; Petramala et al. 2012; Rhodes et al. 2015]. Low bone mineral density was reported in NF1 [Brunetti-Pierri et al. 2008; Dulai et al. 2007; Stevenson et al. 2007; Yilmaz et al. 2007; Armstrong et al. 2013]. Moreover, recent studies demonstrated in vitro bone cell anomalies consisting in increased proliferation, decreased osteoblasts differentiation, and excessive osteoclasts resorption activity in $Nf1^{+/-}$ mices. NF1 haploinsufficiency determines the hyperactivation of the RAS/MAPK signaling cascade, also observed in the gain-of-function mutations of the other Rasopathies [Alanne et al. 2012; Heervä et al. 2012; Sharma et al. 2013; Rhodes et al. 2015]. High levels of urinary resorption markers were found in patients with RAS/MAPK disorders consistent with increased osteoclast activity [Stevenson et al. 2011]. As concerns NS, literature data are particularly scanty [Takagi et al. 2000; Noordam et al. 2002; Stevenson et al. 2011; Choudhry et al. 2012]. In NS and in all other rarer Rasopathies, bone density appraisal is critical as measurements are expected to be affected and possibly confounded by other aspects of the syndromes: retarded bone maturation, delayed pubertal development, and short stature. Therefore, correction for these parameters is relevant and needs to be employed when assessing bone status. The point is indeed to determine whether low bone mineralization is primary or secondary to the syndromic characteristics. For this reason, we decided to evaluate our cohort by QUS, which parameters are standardizable for those variables [Baroncelli et al. 2006; Baroncelli 2008; Mussa et al. 2012]. As a second point, QUS exploits different perspectives compared to radiological methods, exploring the skeletal tissue by mechanical

solicitation and providing additional information. Ultrasound explore mineralization, connectivity, mineral density, micro-architecture, and structural characteristics which are highly correlated with skeletal biomechanical resistance [Baroncelli, 2008]. These aspects may be crucial in the evaluation of bone anomalies in complex diseases as genetic syndromes. Although several skeletal sites can be explored by QUS methods (e.g., heel, radius, tibia), the phalangeal site is considered optimal as made up of approximately 60% of cortical and 40% trabecular bone, allowing, therefore an appraisal of both skeletal compounds which can be differently affected [Baroncelli, 2008]. The phalangeal device, we used, assesses the skeletal condition by means of two parameters: AD-SoS—the speed of the ultrasound wave, mostly reflecting bone density and elasticity [Baroncelli, 2008; Guglielmi et al. 2010a,b] and BTT—the time of transmission of the ultrasound signal—also closely related to cortical thickness [Barkmann et al. 2000; Montagnani et al. 2002; Sakata et al. 2004]. BTT is more reliable and has a higher accuracy than AD-SoS in identifying bone damage, presumably because of its exclusive dependency on bone properties [Sakata et al. 2004; Guglielmi et al. 2010b]. Conversely, AD-SoS has been shown to be affected by the thickness of the tissue surrounding phalanges, being, therefore influenced by the patients' BMI [Sakata et al. 2004]. Our data are consistent with the hypothesis that bone tissue alteration in NS is primary. Actually, all BTT SDS were below the reference range in spite of several corrections attempted: weight, height, BMI, pubertal development, and bone maturation. On the opposite, AD-SoS SDS were normal, probably because this parameter is largely affected by the thickness of the soft tissue surrounding the phalanges (i.e., depends on the BMI). Thin individuals, as typically NS patients are, have consistently overestimated bone measurements leading to a falsely normal appraisal, as already observed in similar studies [Mussa et al. 2012].

This hypothesis is confirmed by the fact that after BMI-correction AD-SoS values fall under normal values, overlapping the measurement obtained by BTT. The latter is independent of soft tissue thickness and is actually considered the most indicative parameter of the real skeletal condition. In our cohort, 25% of the patients show reduced QUS measurements for their age based on BTT. In spite of the correction attempts employed to minimize the influence of confounding factors, QUS measurements indicate that bone impairment persists, evidencing overall low measurements in nearly 15% of the cohort. Serum and urinary biochemical bone markers commonly employed in clinical practice were normal. In particular, the vitamin D-PTH-calcium metabolism markers were within the normal range, and did not show correlation with bone measurement. Based on these observations, our results indicate that bone impairment in NS is likely primary. This hypothesis has been already proposed by Choudhry et al. [Choudry et al. 2012] who also found a low bone mineralization in NS patients without evidence of abnormalities in bone metabolism markers, as in our study. The main limitation of this study is in the small number of patients and the observational design which prevent to draw any conclusion concerning whether the bone impairment observed is related to skeletal fragility. The number of fractured patients observed seems rather to reflect that observed in the general population, therefore questioning the clinical implication of the bone damage detected by QUS in childhood. Longitudinal studies are needed to evaluate the progression of the skeletal impairment and the actual long-term fracture rate in NS individuals, especially in adulthood and elderly.

Chapter 7.

Cardiac involvement: the CARNET study

This work has been published as

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BACKGROUND

Cardiovascular involvement is one of the most diagnostic and prognostic feature of Rasopathies. A wide spectrum of congenital heart disease has been so far reported [Marino et al. 1999, Roberts et al. 2013, Digilio et al. 2013]. Genotype-phenotype correlations have been established, including PS and PTPN11mutations, HCM and RAF1, HRAS and a subset of PTPN11 mutations, and mitral valve defects (MVD) and SHOC2 c.4A > G change [Tartaglia et al. 2002, Sarkozy et al. 2003, Pandit et al. 2007, Zampino et al. 2007, Cordeddu et al. 2009]. To date, limited information are available on genotype, phenotype and clinical/surgical outcomes in these patients. The aim of this study was to extend the present knowledge on patients with Rasopathies, providing data on molecular diagnosis and heart involvement, with a comprehensive assessment of morbidity and mortality, focusing on the impact of the genotype on the clinical outcome.

PATIENTS AND METHODS

Patients - This is a multi-centric, retrospective, observational study conducted in seven cardiac centers (Italy and UK) with expertise in Rasopathies and participating in the CArdiac Rasopathy NETwork (CARNET). The study retrospectively analyzed clinical records of all patients with molecularly confirmed diagnosis of NS, NSML, CS or CFCS, followed up until July 2014. Data about age, sex, clinical and molecular diagnosis, type of heart defect, timing of cardiac procedures and date and cause of death (where appropriate) were collected. Data were centralized in a unique database. The Ethical Committees of all the participating centers approved the study protocol.

Statistical analysis - Data were presented as median and range, mean ± standard deviation, or percentages and CI, as appropriate. Mortality was described as crude mortality. Logistic regression models where used to study how categorical variables (syndrome, presence of any cardiac defect and presence of a specific cardiac defect) were associated with each gene mutation; these analyses were adjusted for sex of the patients. Through Poisson regression, the following associations have been studied: the association between mutated genes and number of procedures received by each patient with a cardiac defect; the association of each syndrome with number of interventions; the association between presence of PS, HCM, AVC and other heart defects, and the number of interventions. Kaplan-Meier (KM) curves were used to describe the incidental risk of intervention and mortality, stratified by time, for each mutated gene, syndrome and heart defects. In the population of subjects with heart defects, Cox proportional hazards models were used to assess the effect of having a mutated gene, a syndrome or a specific heart defect on the risk of intervention, respectively adjusting for sex and heart defect, sex and heart defect, sex and gene. KM models were also used to describe mortality as cumulative survival at 1, 5, 10 and 20 years.

RESULTS

Three hundred seventy-one individuals with a RASopathy were enrolled, including 297 (80.1%) with a clinical diagnosis of NS, 45 (12.1%) with NSML, 22 (5.9%) with CFCS, and 7 (1.9%) with CS. One hundred sixty-five patients (44.5%) were females. Median age at last follow-up was 8.75 years (range 11 days–47.6 years). Two hundred ninety-eight patients (80.3%) had a cardiac involvement. More than half subjects had PS (59%), mostly isolated (81% of all patients with PS). Eighty-one (27%) had HCM;

among these, 32% also had mitral valve dysplasia and/or AVD. Other defects frequently reported included ASD (11%), AVD (10%), VSD (4.7%), AVC (4.4%). Tetralogy of Fallot, aortic coarctation, coronary anomalies and patent duct arteriosus were reported in 3% of the patients. Among all patients with CHD, 141 (47.3%) underwent at least one surgical procedure or catheter intervention. Fifty-eight patients (41.1% of patients who underwent the first procedure) needed a second intervention, the majority (approx. 60%) being patients affected by PS, who underwent a second procedure after a first, unsuccessful treatment (85% of cases, mainly after primary percutaneous balloon pulmonary valvuloplasty). In 7 patients (12%), a significant pleural or pericardial effusion was the reason for an early reintervention. The association between each mutated gene and number of interventions reveals individuals heterozygous for BRAF mutations had a significantly lower number of interventions compared to patients with mutations of other genes (aIRR: 0.448, BCa 95% CI: [0.158, 0.859]). PTPN11 mutations increased the risk of early intervention, showing the strongest difference around 15 years of age. This difference was no longer observed at 20 years of age. Patients with BRAF mutations underwent interventions later, compared to those with heterozygous mutations of other genes. RAF1 and SOS1 did not show a clear difference in terms of risk of intervention. Poisson regression showed that subjects with CFCS had a significantly lower number of interventions, while NS was associated with a significantly higher number of interventions compared to other syndromes, independently from the type of heart defect. Subjects with PS and AVC had a significantly higher risk of earlier interventions. Crude mortality was 0.29/100 patients-year. Cumulative survival was 98.8%, 98.2%, 97.7%, 94.3%, at 1, 5, 10, and 20 years respectively. Restricted estimated mean survival at 20 years of follow-up was 19.6 years. Ten patients died

(2.7% of the entire cohort; 3.4% of patients with cardiac defect). Among them, two patients died from leukemia. Both were affected by NS and carried PTPN11 mutations, with ASD and HCM respectively. For the remaining 8 patients, death occurred due to cardiac causes.

DISCUSSION

The results of this multicentric in-depth study show that patients affected by Rasopathies have an overall good cumulative survival (94.3% estimated survival at 20 years), along with a higher risk for cardiac events in infants (< 2 y.o.) and young adults with PTPN11 mutations. According to the literature, PS was the most common heart defect in this study population, followed by HCM and ASD. Among patients affected with a heart disease, almost half underwent a percutaneous and/or surgical intervention. These results are in agreement with previous studies who reported an intervention in a half of their NS population, with 65% of patients requiring additional interventions [Prendiville et al. 2014]. In patients with HCM and NS with a symptomatic left ventricular outflow obstruction requiring an intervention, myectomy has been demonstrated as feasible and a practical alternative to heart transplant. In this cohort, patients with classic NS phenotype were most likely to receive a higher number of percutaneous or surgical interventions, while CFCS patients received a lower number of interventions, in agreement with the negative association between BRAF and the risk of reintervention. Mortality is relatively low in patients affected by Rasopathies. We observed a mortality of 2.7% in the entire cohort, and of 3.4% among patients with cardiovascular involvement. Overall, our data document that heart defects and associated complications were the most recurrent cause of death in our Rasopathy cohort. In particular, NSML or NS patients with HCM, due to PTPN11

gene mutations, were at higher risk of cardiac death. Indeed, out of 7 patients with HCM associated with NS or NSML, 6 died for cardiac causes. Data from the Pediatric Cardiomyopathy Registry showed that crude mortality was worse in syndromic HCM (mainly, NS) compared to non-syndromic HCM [Colan et al. 2007, Wilkinson et al. 2012]. In conclusion, this multicentric study, based on a large cohort of patients with molecular confirmed diagnosis of Rasopathy, provides specific information on cardiac morbidity and mortality in these patients. Percutaneous or surgical intervention is required in almost half of the individuals affected by Rasopathies, particularly in subject with NS and PS while, the number of interventions is considerably lower among those with CS and CFCS. Cardiac mortality is relatively uncommon in these disorders. However, the association between HCM, PTPN11 mutations and NSML, with a peculiar distribution of age (infants and young adults), seems to represent a risk factor for surgical mortality or sudden death.

Conclusive remarks

In the last years, in collaboration with national and international prestigious centers, we had the opportunity to broaden and deepen some clinical and molecular aspects of Noonan syndrome. An extreme clinical variability characterizes this condition underlying the necessity of a multidisciplinary approach to the patients. The continuous identification of several genes responsible for Noonan syndrome and the related clinical conditions, has allowed to define in an increasingly detailed manner the genotype-phenotype correlations, in order to outline targeted follow up strategies and potential treatments for these disorders. However, in approximately 20% of patients with a suggestive phenotype of a Rasopathy, no mutations in the known causative genes have been identified so far. The expansion in understanding the biochemical complexity of Ras signaling, together with the availability of new sophisticated molecular technologies, such as exome and genome sequencing, will lead to deepen the molecular pathogenetic mechanisms of the Rasopathies and to better define the clinical implications and the follow up strategies for these congenital disorders.

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