Molecular and clinical profile of patients referred as Noonan or Noonan-like syndrome in Greece: a cohort of 86 patients

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Abstract
Noonan syndrome (NS) is an autosomal dominant disorder characterized by clinical and genetic heterogeneity. It belongs to a wider group of pathologies, known as Rasopathies, due to the implication of genes encoding components of the Ras/MAPK signalling pathway. Recording the genetic alterations across populations helps assessing specific features to specific genes which is essential for better disease’s recognition, prognosis and monitoring. Herein, we report the clinical and molecular data of a Greek cohort comprising of 86 NS or NS-like patients admitted at a single tertiary Centre in Athens, Greece. The analysis was performed using Sanger and next-generation sequencing, comprising 14 different genes. The mutational rates of the confirmed NS-associated genes in the Greek NS population are as follows: PTPN11 32.5%; RIT1 5.8%; SOS1 4.7%; BRAF 1.2%; CBL 1.2%; KRAS 1.2%; MAP2K1 1.2%; RAF1 1.2%; SHOC2 1.2%, corresponding to 50% of positivity in total NS population. The genotype–phenotype analysis showed statistically significant differences in craniofacial dysmorphisms \( (p = 0.005) \) and pulmonary valve stenosis (PS) \( (p < 0.001) \) frequencies between patients harbouring a pathogenic variant and patients without pathogenic variant in any of the tested genes. Patients with at least a pathogenic variant had 6.71 times greater odds to develop PS compared to pathogenic variant-negative patients \( (OR = 6.71, 95\% CI = (2.61, 17.27)) \). PTPN11 positive patients showed higher frequency of epicanthal folds \( (p = 0.004) \), ptosis \( (p = 0.001) \) and coarseness \( (p = 0.001) \) and lower frequency of neurological findings \( (p = 0.006) \), compared to patients carrying pathogenic variants in other genes.

Conclusion: Craniofacial dysmorphism and PS prevail among pathogenic variant positive compared to pathogenic variant negative NS and NS-like patients while neurological defects are less common in PTPN11-affected NS patients compared to patients harbouring pathogenic variants in other genes. The significant prevalence of the Ras/MAPK pathogenic variants (17.4%), other than PTPN11, in Greek NS patients, highlights the necessity of a wider spectrum of molecular diagnosis.

What is Known:
- Noonan syndrome (NS) has been associated with pathogenic variants in molecules-components of the Ras/MAPK pathway.
- Clinical and genetic description of NS patients worldwide helps establishing personalized monitoring.

What is New:
- NS and NS-like mutational rate in Greece reaches 50% with pathogenic variants identified mostly in PTPN11 (32.5%), RIT1 (6%) and SOS1 (4.7%) genes.
- The risk for pulmonary stenosis increases 6.71-fold in NS patients with a pathogenic variant compared to patients without genetic alterations.

Keywords Noonan syndrome · Rasopathies · PTPN11 · Ras/MAPK pathway · Heart defects · Craniofacial dysmorphism

Abbreviations
CFC Cardiofacio-cutaneous syndrome
HCM Hypertrophic cardiomyopathy
NS Noonan syndrome
PS Pulmonary valve stenosis
NGS Next-generation sequencing
SNPs  Single-nucleotide polymorphisms
VUS  Variants of unknown clinical significance

Introduction

Noonan syndrome (NS) (OMIM 163950) is an autosomal dominant disorder with marked variability either during one patient’s lifetime. The estimated worldwide frequency ranges between 1/1000–2500 births, although milder expression is thought to occur in 1/100 live births [1]. The syndrome has a multi-system involvement with key features including typical craniofacial dysmorphic characteristics and congenital heart defects, most commonly pulmonary valve stenosis (PS) [2]. Skin pigmentation, lymphatic abnormalities, cryptorchidism, ocular findings, skeletal defects, failure to thrive, developmental delay and coagulation defects may also be present. Increased risk of developing haematological cancers and cases of rhabdomyosarcoma have also been reported [2, 3].

NS development has been associated with dysregulation of the Ras/MAPK signalling pathway, one of the central molecular pathways regulating proliferation, differentiation and cell’s survival [4]. Alterations resulting in instability of its molecule-components favour their permanent activation, thus, leading to uncontrolled signalling downstream, affecting multiple organs and contribute to the development of cell proliferative disorders, such as cancer. Pathologies associated with germline pathogenic variants in genes encoding members of Ras/MAPK pathway present overlapping phenotypes and are grouped under the term of “Rasopathies” [5], with NS being the most common.

Among the members of this molecular network, heterozygous pathogenic variants in the PTPN11 gene account for the majority of all NS cases [6]. NS patients negative for PTPN11 pathogenic variants may exhibit a pathogenic variant or a variant of uncertain clinical significance (VUS) in a subset of genes encoding downstream regulators and effectors of Ras/MAPK pathway, while the role of other downstream genes such as A2ML1 [7–9] is still under investigation [10, 11]. Variable mutational rates have been recorded, depending on the number of participants and on the method of patients’ recruitment and evaluation [12, 13].

Extended use of next-generation sequencing (NGS) techniques during the last decade helps unfolding the heterogeneous genetic basis of NS. The discovery of new variants and new altered genes in NS/NS-like patients highlights potential therapeutic targets [14] and, in combination with specific features, contributes to establish accurate diagnosis and monitoring. Thus, germline pathogenic variants in HRAS have been associated with Costello syndrome [MIM 218040] [15]; in BRAF, KRAS, MAP2K1 and MAP2K2 with cardiofacio-cutaneous (CFC) syndrome [MIM 115150] [16] and in SPRED1 with Legius syndrome [MIM 611431] [17]. In clinical practice, however, it is often complicated to distinguish syndromes with overlapping phenotypes, especially in young age, as both severity and clinical heterogeneity make the diagnosis challenging.

Our group had previously published PTPN11 analysis by Sanger sequencing in 60 individuals clinically characterized as NS/NS-like Greek patients [18]. The current study expands the genetic analysis in 14 Rasopathy-associated genes using NGS and presents a more comprehensive molecular and clinical profile of this cohort with inclusion of 26 more patients, along with genotype–phenotype correlations. The genetic testing of the additional genes increases the mutational rate in the NS Greek population confirming the necessity of molecular diagnosis with accurate diagnostic tools.

Materials and methods

Patients

The study’s cohort consists of 86 Greek neonates, children and teenagers recruited during 2005–2018, with the clinical suspicion of NS or NLS. Among them, 48 patients had been previously found negative for PTPN11 pathogenic variants, and 16 had no molecular testing. The clinical and molecular characteristics of 22 PTPN11 patients (presented in our previous work [18] and recruited during the same period) were also presented and included in statistical analysis of the total Greek NS population. Patients with phenotypic similarities to NS were referred by paediatricians (38/86, 44%), cardiologists (29/86, 34%), endocrinologists (12/86, 14%), neurologists (5/86 6%) and dermatologists (2/86, 2%) to our outpatient clinics and were further assessed by a clinical geneticist at the Department of Medical Genetics of Athens University Medical School for evidence of major or minor anomalies. All phenotypes were systematically evaluated and registered by clinical geneticists at the Department of Medical Genetics of the National and Kapodistrian University of Athens. In every patient referred, all organic system’s impairment which are involved in NS has been checked as cardiological, endocrine, hematology, urinary, skeletal, skin, hearing, vision and dental. Dysmorphic features were thoroughly itemized and detailed family history as well as a karyotype was undertaken in each case. Patients that fulfilled the criteria developed by Van der Burgt et al. [19] (Table 1) as well as individuals with a highly suggestive phenotype who lacked a definitive NS diagnosis were referred consecutively to the Research Lab of the 3rd Department of Pediatrics, of the National and Kapodistrian University Athens, for molecular investigation. Major and minor criteria of NS
are depicted in Table 1. In this scoring system, patients are first classified according to the facial features (typical or suggestive); and if “typical”, the diagnosis of NS is established in the presence of any other major sign (or two minor signs), while in cases with “suggestive” NS face, there is need of two major (or three minor) criteria to confirm the NS diagnosis.

**Molecular study**

Genomic DNA was isolated from peripheral blood lymphocytes (NucleoSpin Blood, Macherey–Nagel, Duren, Germany). All samples were tested for 14 RASopathy-associated genes using NGS except from the 22 patients previously found positive for PTPN11 pathogenic variants. Targeted analysis referred to PTPN11 (NM_002834.5), SHOC2 (NM_007373.4), RAF1 (NM_002880.4), BRF (NM_001374258.1), KRAS (NM_004985.5), NRAS (NM_002524.5), SOS1 (NM_005633.4), PTPN11 (NM_007373.4), SHOC2 (NM_005343.4). NGS was performed using the Ion S5 system (Thermo Fisher Scientific). Sequence reads were aligned to the reference sequence (GRCh38) Ion reporter, followed by variant calling using Noonan workflow version 5.10. Sanger sequencing was used to confirm all clinically significant variants and fill in regions with insufficient coverage (horizontal < 100%; vertical < 30%).

**Statistical analysis**

Clinical and molecular description of NS Greek population, as well as statistical analyses, was conducted for all 86 NS patients referred to our hospital. Initial statistical analysis was based on descriptive statistics. Categorical variables including pathogenic variants and polymorphisms were described by absolute and relative frequencies, while for continues variables, mean, standard deviation, median, minimum, and maximum were provided. The association between clinical features and pathogenic variants/polymorphisms was assessed by Fisher’s exact test or chi-square test where the assumptions were met. Multiple comparison adjustment was necessary due to the various clinical features to be tested across several pathogenic variants and polymorphisms. Each pathogenic variant/polymorphism was tested for association with 25 clinical features; thus, statistical significance level was set to $\alpha = 0.05/25 = 0.002$ (Bonferroni correction). Statistically significant association between pathogenic variants and polymorphisms was not found. Entire statistical analysis was conducted in STATA 15.1 and all tests were two-sided.

**Results**

**Molecular study**

In the total Greek NS cohort, 50/86 cases (58%) were positive for pathogenic variants in, at least, one of the Rasopathy-associated genes tested. Among them, 28 patients (32.5%) were PTPN11 positive presenting 13 different PTPN11 pathogenic variants, with p.Tyr63Cys (6/28 cases; 22%); p.Asn308Asp (5/28 cases; 17%) and p.Met504Val (4/28 cases; 14%) being the most frequent. Among the PTPN11 negative patients (58/86), targeted sequencing of all 14 RASopathy-associated genes included in NGS panel revealed 22 more patients (25.8%) with pathogenic or likely pathogenic variants. The most frequently altered reported genes were RIT1 (7 patients), A2ML1 (3 patients), SOS1 (3 patients) and SPRED1 (2 patients). Two patients were compound heterozygotes; one (N022) carried a pathogenic variant in PTPN11 and a VUS in SPRED1 (p.Glu139Asp and p.Arg334Cys, respectively), and the second (N030) carried a VUS in A2ML1 and a pathogenic variantin SOS1 (p.Arg657His and p.Met269Thr, respectively). Pathogenic variants in BRAF, CBL, KRAS, MAP2K1, RAF1 and SHOC2 were found in 6 different patients while no pathogenic variant was identified in MAP2K2, NRAS or HRAS. Supplementary Table S1 resumes molecular and clinical data of each patient with pathogenic, likely pathogenic variants or VUS in all genes. All variants were heterozygous
missense changes, except for c.1096-4_1096-1del in the CBL gene. One novel variant was identified in RIT1 gene (c.235C > G; p.Gln79Glu) (N013), which has been recently reported in public databases (dbSNP138, ExAC and 1000 genomes); however, no clinical symptoms have been provided. According to PolyPhen analysis (PolyPhen-2 v2), this variant is predicted to be damaging (score 1.00). N002 carried the c.4076G > A (p.Arg1359His) in the A2ML1 gene which is characterized as VUS or likely benign. The patient presented with craniofacial dysmorphisms, short stature and cryptorchidism. However, as A2ML1 has not been confirmed as a NS causative gene, we excluded A2ML1 positive samples from further statistical analysis, as well as patients with a SPRED1 pathogenic variant which have been associated with Legius syndrome. We also excluded from statistical analysis the patient that was positive for a RIT1 variant characterized as VUS and the patient with the novel RIT1 variant. Thus, the mutational rates of the confirmed NS-associated genes in the Greek NS population are as follows: PTPN11 32.5%; RIT1 5.8%; SOS1 4.7%; BRAF 1.2%; CBL 1.2%; KRAS 1.2%; MAP2K1 1.2%; RAF1 1.2%; SHOC2 1.2%, corresponding to 50% of positivity in total NS population.

Presenting symptoms and clinical features of patients

Table 2 shows patients’ clinical features. In the total population mean age at diagnosis was 4.4 ± 4.7 years (1 month to 19 years) and male patients prevailed to female (56 vs. 30). In all patients, variable combinations of craniofacial abnormalities were recorded such as microcephaly, epicanthal folds, ptosis, low nasal bridge, hypertelorism, down slanting palpebral fissures, low set posteriorly rotated ears, coarseness and webbed/thick neck. Craniofacial dysmorphisms (98.8%) and short stature (67.4%) were the most frequent characteristics of the patients, while PS was the most prevalent among congenital heart defects (47.7%). Neurological and skeletal defects were observed in 39.5% and 46.5% of patients, respectively, and cryptorchidism in 25%. No patient presented with seizures, lymphatic dysplasia, bleeding diathesis, cancer or macrocephaly, while sensorineural hearing loss, lymphedema or kidney disorders were rarely reported. Seven patients had a positive family history. As shown in Table 3, most patients showed with typical face and heart defect (49%).

The comparison between pathogenic variant-positive and-negative patients revealed a higher frequency of heart disease (p = 0.016) and especially for PS (p < 0.001) (only 3 patients had hypertrophic cardiomyopathy, HCM) in patients who had a pathogenic variant in any of the tested genes compared to pathogenic variant-free patients. Specifically, patients with at least one pathogenic variant had 6.71 times greater odds to develop PS compared to patients without pathogenic variant detected (OR = 6.71, 95%; CI = (2.61, 17.27)). Epicanthal folds, ptosis, low nasal bridge hypertelorism, down slanting palpebral fissures, low set rotated ears, coarseness and webbed neck were more frequently observed in pathogenic variant positive patients compared to negative ones (p < 0.001 for all features). Additionally, the phenotypical combination of typical face and heart disease (A1 + 2A, Table 3) was found in 65% of pathogenic variant-positive patients and in 33% of pathogenic variant-negative patients. No other significant correlation was found with respect to presence of growth and skeletal anomalies.

Regardless genotype-phenotypes associations, we compared patients’ phenotypes harbouring a pathogenic variant in a specific gene with patients harbouring a pathogenic variant in any other gene. We observed a higher frequency of epicanthal folds (p = 0.004), ptosis (p = 0.001), down slanting palpebral fissures (p = 0.024) and coarseness (p = 0.001) in PTPN11 positive patients compared to pathogenic variant-positive patients in other genes. On the other hand, intellectual disability of variable degree was less frequent in PTPN11 positive patients compared with pathogenic variant-positive patients in other genes (p = 0.008).

The record of single nucleotide polymorphisms (SNPs) showed 27 different exonic SNPs, with A2ML1 accounting for most of them (Table 4). Their frequencies varied from 1.6 to 100% in the total population. Variant c.660C > A in MAP2K2 gene was observed in patients without neurological features or downward eye slant (OR = 0.083, 95% CI = (0.02, 0.34); OR = 0.11, 95% CI = (0.02, 0.45), respectively) (data not shown). Thirty-three (33/36) pathogenic variant-negative patients vs 17(17/50) pathogenic variant-positive patients carried this polymorphism. No other correlation was found between a specific SNP and any clinical feature or pathogenic variant. It should be noted that all polymorphisms were also detected in patients who did not fulfil NS criteria.

Discussion

The phenotype of Noonan and Noonan-like syndromes are recognised, or suspected, at birth, by experienced paediatricians and cardiologists and, later in life, by other specialties, such as neurologists, endocrinologists or dermatologists. Due to the overlapping phenotypes with other entities and because of the subsequent changing craniofacial features during life, it is often difficult to obtain a definite diagnosis based only on clinical criteria. The genetic analysis may help in ending the diagnostic odyssey of the patient and, sometimes, guides medical decisions for monitoring and therapy. In the case of NS and NS-like patients, the recording of mutational detection rates in different cohorts worldwide shows a great variation, depending on the clinical
| Patients’ characteristics | Total population (N = 86) | Patients positive for a pathogenic variant (N = 43) | Patients negative for a pathogenic variant (N = 43) | Patients positive for a PTPN11 pathogenic variant patients (N = 28) | Patients positive for pathogenic variant in gene other than PTPN11 (N = 15) |
|---------------------------|---------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Age at diagnosis; years (mean ± SD) | 4.4 ± 4.7 | 4.61 ± 5.0 | 4.21 ± 4.4 | 5.34 ± 5.7 | 3.2 ± 3.1 |
| Sex; N (male vs female) | 56/30 | 23/20 | 33/10 | 17/11 | 6/9 |
| p = 0.024 |
| Craniofacial dysmorphism; N (%) | 85 (98.8) | 43 (100) | 42 (97.7) | 28 (100) | 15 (100) |
| Microcephaly; N (%) | 8 (9.3) | 5 (11.7) | 3 (7.0) | 3 (10.7) | 2 (13.3) |
| Epicanthal folds; N (%) | 33 (38.4) | 30 (69.8) | 3 (7.0) | 24 (85.7) | 6 (40.0) |
| p < 0.001 |
| Ptosis; N (%) | 30 (34.8) | 29 (67.4) | 1 (2.3) | 24 (85.7) | 5 (33.3) |
| p < 0.001 |
| Low nasal bridge; N (%) | 34 (39.5) | 31 (72.1) | 3 (7.0) | 23 (82.1) | 8 (53.3) |
| p < 0.001 |
| Hypertelorism; N (%) | 36 (41.9) | 31 (72.1) | 5 (11.6) | 21 (75) | 10 (66.7) |
| p < 0.001 |
| Down slanting palpebral fissures; N (%) | 28 (32.6) | 25 (58.1) | 3 (7.0) | 20 (71.4) | 5 (33.3) |
| p < 0.001 |
| Low set/posteriorly rotated ears; N (%) | 36 (41.9) | 32 (74.4) | 4 (9.3) | 23 (82.1) | 9 (60.0) |
| p < 0.001 |
| Papillomas; N (%) | 1 (1.2) | 1 (2.3) | 0 | 1 (3.6) | 0 |
| p = 0.004 |
| Coarseness; N (%) | 46 (53.5) | 31 (72.1) | 15 (34.9) | 25 (89.3) | 6 (37.5) |
| p = 0.004 |
| p < 0.001 |
| Webbed/thick neck; N (%) | 36 (41.9) | 32 (74.4) | 4 (9.3) | 23 (82.1) | 9 (60.0) |
| p < 0.001 |
| Heart disease; N (%) | 62 (72.1) | 36 (83.7) | 26 (60.5) | 23 (82.1) | 13 (86.7) |
| p = 0.016 |
| Pulmonary stenosis; N (%) | 41 (47.7) | 30 (69.8) | 11 (25.6) | 20 (71.4) | 10 (66.7) |
| p = 0.001 |
| Hypertrophic myopathy; N (%) | 5 (5.8) | 3 (7.0) | 2 (4.7) | 0 | 3 (20.0) |
| p = 0.001 |
| Other cardiological problems; N (%) | 17 (19.8) | 9 (20.9) | 8 (18.6) | 5 (17.9) | 4 (26.7) |
| p = 0.037 |
| Skeletal features; N (%) | 40 (46.5) | 18 (41.9) | 22 (51.2) | 11 (39.3) | 7 (46.7) |
| Low height; N (%) | 58 (67.4) | 31 (72.1) | 27 (62.8) | 19 (67.9) | 12 (80.0) |
| Skin manifestations; N (%) | 7 (8.1) | 4 (9.3) | 3 (7.0) | 1 (3.6) | 3 (20.0) |
| Neurological features; N (%) | 34 (39.5) | 14 (32.6) | 20 (46.5) | 5 (17.9) | 9 (60.0) |
| p = 0.008 |
| Others | | | | | |
| Hearing loss; N (%) | 1 (1.2) | 1 (2.3) | 0 | 1 (3.6) | 0 |
| Cryptorchidism; N (%) | 14 (25) | 7 (30.4) | 7 (21.2) | 2 (11.8) | 5 (83.3) |
| Kidney disorders; N (%) | 4 (4.7) | 2 (4.7) | 2 (4.7) | 0 | 2 (13.3) |
| Lymphedema; N (%) | 4 (4.7) | 3 (4.7) | 1 (2.3) | 3 (10.7) | 0 |
| Family history; N (%) | 7 (8.1) | 7 (14) | 0 | 5 (17.9) | 2 (13.3) |
inclusion criteria stringency, the number of participants and the genetic testing applied [2, 11]. According to the published results, PTPN11 pathogenic variants account for ~50% of cases, whereas the analysis of other Ras/MAPK related genes increases the mutational rate up to 70–80% [11, 20–22].

| Scoring | Total population (N = 86) | Patients positive for a pathogenic variant (N = 43) | Patients negative for a pathogenic variant (N = 43) | Patients positive for a PTPN11 pathogenic variant (N = 28) | Patients positive for a pathogenic variant in gene other than PTPN11 (N = 15) |
|---------|---------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|         | n  | %        | n  | %        | n  | %        | n  | %        | n  | %        |
| 1A + 2A | 42 | 48.84    | 28 | 65.12    | 14 | 32.56    | 19 | 67.86    | 9  | 60.00    |
| 1A + 3A | 9  | 10.47    | 5  | 11.63    | 4  | 9.30     | 5  | 17.86    | 0  | 0.00     |
| 1A + 4A | 8  | 9.30     | 3  | 6.98     | 5  | 11.63    | 2  | 7.14     | 1  | 6.67     |
| 1A + 2B + 3B | 3 | 3.49 | 1 | 2.33 | 2 | 4.65 | 1 | 3.57 | 0 | 0.00 |
| 1A + 2B + 6B | 5 | 5.81 | 1 | 2.33 | 4 | 9.30 | 0 | 0.00 | 1 | 6.67 |
| 1A + 3B + 5B | 1 | 1.16 | 1 | 2.33 | 0 | 0.00 | 0 | 0.00 | 1 | 6.67 |
| 1A + 3B + 6B | 6 | 6.98 | 0 | 0.00 | 6 | 13.95 | 0 | 0.00 | 0 | 0.00 |
| 1B + 2A + 3A | 2 | 2.33 | 0 | 0.00 | 2 | 4.65 | 0 | 0.00 | 0 | 0.00 |
| 1B + 2A + 4A | 2 | 2.33 | 1 | 2.33 | 1 | 2.33 | 0 | 0.00 | 1 | 6.67 |
| 1B + 2B + 3B + 4B | 1 | 1.16 | 0 | 0.00 | 1 | 2.33 | 0 | 0.00 | 0 | 0.00 |
| 1B + 2B + 3B + 6B | 1 | 1.16 | 1 | 2.33 | 0 | 0.00 | 1 | 3.57 | 0 | 0.00 |
| 1B + 3A + 4A | 5 | 5.81 | 1 | 2.33 | 4 | 9.30 | 0 | 0.00 | 1 | 6.67 |
| 1B + 3A + 6A | 1 | 1.16 | 1 | 2.33 | 0 | 0.00 | 0 | 0.00 | 1 | 6.67 |

Table 4 Single nucleotide polymorphisms identified in study population

| Polymorphism | Frequency (%) | Polymorphism | Frequency (%) |
|--------------|--------------|--------------|--------------|
| NRAS_c.360G > A | 1.6 | SPRED1_c.291G > A | 95.3 |
| RIT1_c.31G > C | 98.4 | SPRED1_c.1044T > C | 98.4 |
| SHOC2_c.457C > T | 1.6 | MAP2K1_c.255C > T | 1.6 |
| KRAS_c.483G > A | 96.9 | MAP2K1_c.255C > T | 1.6 |
| KRAS_c.643T > C | 32.8 | MAP2K1_c.711G > A | 1.6 |
| A2ML1_c.186C > T | 9.4 | MAP2K1_c.804C > G | 1.6 |
| A2ML1_c.196C > T | 1.6 | MAP2K2_c.192C > T | 12.5 |
| A2ML1_c.619G > C | 1.6 | MAP2K2_c.453C > T | 39.1 |
| A2ML1_c.1101T > C | 1.6 | MAP2K2_c.660C > A | 78.1 |
| A2ML1_c.1109T > C | 1.6 | MAP2K2_c.1211C > T | 1.6 |
| A2ML1_c.1275A > G | 17.5 | MAP2K2_c.1212G > A | 3.1 |
| A2ML1_c.2367G > A | 98.4 | MAP2K2_c.1222C > T | 3.1 |
| A2ML1_c.2550C > A | 98.4 | MAP2K2_c.1227C > T | 25 |
| A2ML1_c.2868C > T | 6.3 | SOS1_c.553A > G | 1.6 |
| A2ML1_c.2909G > A | 40.6 | SOS1_c.570C > T | 1.6 |
| A2ML1_c.3237T > A | 50 | SOS1_c.984C > T | 1.6 |
| A2ML1_c.3364C > T | 57.8 | SOS1_c.1964C > T | 3.2 |
| A2ML1_c.3569C > T | 15.6 | SOSI_c.2376A > G | 100 |
| A2ML1_c.3676_3677del | 17.5 | RAF1_c.171T > C | 1.6 |
| A2ML1_c.3686A > G | 98.4 | BRAF_c.723G > A | 1.6 |
| A2ML1_c.3769A > G | 92.2 | BRAF_c.1462A > C | 1.6 |
| A2ML1_c.3843T > C | 8.1 | BRAF_c.1929A > G | 46.9 |
| A2ML1_c.4020A > G | 95.3 | BRAF_c.2128-2A > TA | 1.6 |
In Greece, the genetic analysis depicted 52 heterozygous pathogenic/likely pathogenic variants in 11 out of 14 genes tested in 86 NS patients, with PTPN11, RIT1 and SOS1 presenting the higher frequency. The current knowledge allows the recognition of the genetic basis of more NS patients. The screening of more Ras/MAPK related genes increased the NS and NS-like mutational rate in Greece from 28%, reported in our previous work [18] including only PTPN11, to 51.4% in the current paper. Taking into consideration that A2ML1 gene has not been confirmed as a NS gene and that SPRED1 has been associated with Legius syndrome, the mutational rate of NS patients in Greece is formed as of 50%. Phenotypically, our findings confirmed those observed in other cohorts with craniofacial dysmorphisms, and pulmonary stenosis (PS) being significantly associated with the presence of pathogenic variants in any of the tested genes [2, 20].

PTPN11 pathogenic variants were observed in 32.5% of our population. The risk for pulmonary stenosis increased 5.13-fold (OR: 95% CI=(1.91, 13.76)) in NS patients with a PTPN11 pathogenic variant compared to patients without pathogenic variant while intellectual disability of variable degree was less frequent in PTPN11 positive patients compared to patients with a pathogenic variant in another gene tested. All PTPN11 variants found were missense and favour the open configuration of the enzyme allowing its permanent catalytic function [6, 23]. Among the more frequent variants, p.Asn308Asp has been also recorded as the most prevalent in other populations, reaching ~20% in Europe, USA, Argentina [21, 24], Brazil [25] and 11% in South India [12].

It has been suggested that RIT1-mutated NS patients present higher rates of HCM compared to other NS patients [13, 26]. Although RIT1 was the second most frequently altered gene in our cohort, detected in 6% of total NS population, we could not confirm the above clinical relevance because of the rarity of patients with HCM in our population. The detected mutational rate is similar to previous studies [13, 27, 28] worldwide (4–9%). All but one variants detected have already been described in the literature and cluster at codons encoding highly conserved amino acids in functionally important domains. However, the most commonly observed pathogenic variants in other cohorts — at positions 57 and 82 of the protein — [13, 28, 29] were absent from our population. The most frequent variant was p.Ser35Thr (3/5 of RIT1 patients) which has been associated with activated MAPK signalling [13]. The identified novel variant p.Gln79Glu corresponds to an oncogenic RAS alteration p.Gln61Glu, implicated in the activation of MAPK signalling in a cell-specific manner [13]. Phenotypically, this patient (N0880) presented craniofacial dysmorphisms, webbed/thick neck, intellectual disability, cryptorchidism and short stature. According to ACMG guidelines, the in vitro functional studies supportive of a damaging effect of the p.Gln79Glu variant on the gene product and its absence from control subjects in the population databases together with pathogenicity prediction and clinical signs provide strong evidence for the causality of this variant.

Four patients (4.7%) of our cohort presented 4 different variants in the SOS1 gene [2], the second-most-common genetic cause of NS worldwide, accounting for ~15% of NS cases [29–31]. All variants observed in our patients have been previously described [30, 32] and reside in regions that may disrupt the autoinhibited protein’s conformation [30]. The associated phenotypes of our patients were clearly in NS spectrum, without the specific features attributed to SOS1 pathogenic variants such as ectodermal features and macrocephaly [30].

Among the other genes tested, pathogenic variants were observed in BRAF, CBL, KRAS, MAP2K1 (MEK1), RAF1 and SHOC2 at a lower frequency (1.2%). Germline variants in KRAS and BRAF genes have been associated with NS and CFC with a variable clinical phenotype depending on the affected residue, while somatic pathogenic variants are closely related to myeloid malignancies (KRAS), melanoma (BRAF) and other cancers [33–35]. Our patients with heterozygous either KRAS or BRAF pathogenic variants showed typical NS features. KRAS p.Asp153Val variant has already been published as de novo alteration associated with NS and CFC [33, 35, 36] and it is characterized as pathogenic. On the other hand, it has been shown that the observed BRAF variants p.Glu501Gly does not enhance ELK-dependent transcription similar to other pathogenic variants identified in the kinase domain of the protein [35]. RAF1, SHOC2 and CBL are downstream effectors of the Ras/MAPK pathway which have been associated with specific clinical features in NS patients; RAF1 has been suggested to cause cardiomyopathy [37] SHOC2 a rare subset of NS with a unique phenotypic feature of loose anagen hair [38, 39] and CBL has been linked to an entity that resembles NS and predispose to juvenile myelomonocytic leukaemia that may follow a variable course [40, 41]. Although pathogenicity has been attributed to all variants observed in our study, these patients presented craniofacial defects, PS but no other specific features.

According to our results, small-scale recording of NS genetic variants may deviate the genotype–phenotype associations that have been proposed for the NS or NLS associated genes, such as the presence of HCM in RIT1 carriers, or macrocephaly and ectodermal manifestations attributed to SOS1 patients with pathogenic variant patients. What complicated more the definite diagnosis and the extraction of solid associations was the detection of genetic variants classified as of VUS in patients fulfilling the van der Burgt criteria. For instance, a patient with craniofacial dysmorphisms, carrier of the RIT1 p.Lys23Arg (VUS) and a patient with typical NS facial characteristics and heart defects but without skin manifestations, revealed the SPRED1 p.Thr196Ile
(VUS). In line with this, four of our patients with a rather mild phenotype and typical NS face, revealed 4 different rare and non-synonymous variants in A2ML1 gene classified as of VUS. Functional studies and detailed clinical information on A2ML1-mutated NS patients reported in previous studies have put up the question of the driver role of A2ML1 in RASopathies [8] as de novo variants have been identified in patients that hardly met the diagnostic criteria [7, 42]. Furthermore, results from in vitro analysis have failed to show an impact of A2ML1 variants on RAS/MAPK activation [7, 42].

For the significant patients’ number with unknown genetic cause or VUS in our cohort, paediatricians and genetists should inform the patients for a long follow up to detect any changes in the phenotype that may change the initial clinical diagnosis and/or to perform a future reanalysis in other genes, recently associated with NS, like LTRZ1, RRAS, SOS2, RASA2, PPP1CB and MRAS, or even of genes from other molecular pathways that may collaborate with the Ras/MAPK pathway, such as mTOR [43]. Moreover, in some cases, functional studies have shown that a detected variant in a NS patient may not stimulate the Ras/MAPK pathway, although he fulfills the diagnostic criteria. It would be possible that other genes or polymorphisms may have an additive or modifying effect on the phenotype, as the c.660C > A in MAP2K2 which was observed in our patients without neurological features. Thus, frequent follow-up of patients even with typical clinical NS diagnosis is essential for their appropriate and successful management. On the other hand, for patients with NS phenotype, the presence of a genetic variant in one of the Noonan genes facilitates the paediatrician’s planning for precise and multidisciplinary clinical guidance and also allows the referral for proper genetic counselling for the family.

It is certain that unravelling NS molecular basis may help discovering new therapeutic targets and personalizing RASopathy monitoring. In particular, drugs that inhibit components of the Ras/MAPK pathway could be useful for treating RASopathy patients. In vivo experiments have shown that MEK inhibitor treatment during the early life stages may reduce or reverse NS defects in Raf1 or Sos1 mutant mice [44, 45]. In humans, it has recently been reported that trametinib, a highly selective reversible allostERIC inhibitor of MEK1/2 activity might limit myocardial hypertrophy [14, 46] in NS patients with RIT1 pathogenic variants, while a complete remodelling of the entire central lymphatic system in a girl with NS and a SOS1 pathogenic variant has recently been reported [47].

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Authors’ contributions GP, AP and EB conducted material preparation, and wrote of the manuscript. Patient’s stratification was performed by SK-TZ, KK, AP, CK-G and VP. AP wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability Raw data are available upon request.

Code availability Not applicable.

Declarations

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki and its later amendments. Medical ethical approval was granted by the local medical ethics committee of Attikon University general hospital with the file number (4492/16.07.2019).

Consent to participate Written informed consent was obtained from the participants or/and the parents of the participating children, as well as patients’ assess where applicable.

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