1 **Digenic inheritance of subclinical variants in Noonan Syndrome patients: an**
2 **alternative pathogenic model?**
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ABSTRACT

Noonan syndrome (NS) is an autosomal-dominant disorder with variable expressivity and locus heterogeneity. Despite several RAS pathway genes were implicated in NS, 20-30% of patients remain without molecular diagnosis, suggesting the involvement of further genes or multiple mechanisms. Eight patients out of 60, negative for conventional NS mutation analysis, with heterogeneous NS phenotype were investigated by means of target resequencing of 26 RAS/MAPK pathway genes. A trio was further characterized by means of whole exome sequencing. Protein modelling and *in silico* prediction of protein stability allowed to identify possible pathogenic RAS pathway variants in four NS patients. A new c.355T>C variant in *LZTR1* was found in patient 43. Two patients co-inherited variants in *LRP1* and *LZTR1* (patient 53), or *LRP1* and *SOS1* genes (patient 67). The forth patient (56) carried a compound heterozygote of *RASAL3* gene variants and also an *A2ML1* variant. While these subclinical variants are singularly present in healthy parents, they co-segregate in patients, suggesting their addictive effect and supporting a digenic inheritance, as alternative model to a more common monogenic transmission. The ERK1/2 and SAPK/JNK activation state, assessed on immortalized lymphocytes from patients 53 and 67 showed highest phosphorylation levels compared to their asymptomatic parents. These findings together with the lack of their co-occurrence in the 1000Genomes database strengthen the hypothesis of digenic inheritance in a subset of NS patients. This study suggests caution in the exclusion of subclinical variants that might play a pathogenic role providing new insights for alternative hereditary mechanisms.

INTRODUCTION

Noonan syndrome (NS) is a RASopathy with autosomal-dominant inheritance and prevalence of 1:1000–2500 (1). NS is characterized by variable expressivity of the phenotype, including craniofacial features such as hypertelorism, downslanting of the palpebral fissures, ptosis, low-set, posteriorly rotated ears, and webbed or short neck (1–3). More than 80% of individuals with NS exhibit cardiovascular involvement, most frequently congenital heart diseases, pulmonary valve stenosis and hypertrophic cardiomyopathy (4). Other clinical manifestations include cryptorchidism, bleeding disorders, mild neurocognitive delay and pectus deformity, and an increased risk of developing myeloproliferative disorders (3,5). Given the considerable variable expressivity, some NS adults are diagnosed after the birth of an affected child (6,7). NS shows significant locus and allelic heterogeneity, since variants causing disease, affecting gene function, involve different genes of the RAS/MAPK (mitogen-activated protein kinase) signal transduction pathway: *PTPN11* (in 40-50% of the patients), *SOS1* (10-20%), *RAF1* (3-17%), *RIT1* (9%), and the less common *KRAS*, *NRAS*, *BRAF*, *SHOC2*, *MAP2K1*, *CBL, LZTR1, SOS2, RRAS, CDC42* and *A2ML1* (8,9) (1-5%) (9). The *A2ML1* variants have been reported to be associated to few NS patients (10) even if other authors interpreted one of the two previously reported *A2ML1* pathogenic variants, of unknown significance (VUS) (11). Differently, a significant number of data evidenced the relevance of functional role of *LZTR1* in RAS signaling considering the effects of its functional variant in NS patients (9,12), Interestingly, the finding of biallelic *LZTR1* pathogenic variants reported in 12 families with NS children, supports an autosomal recessive inheritance besides the more frequent dominant inheritance pattern with different implications for the NS pathogenesis ~~(~~(13)~~28)~~. Moreover, a few

79 NS and NS–like cases have been reported to show Copy Number Variations (CNV)
80 encompassing NS-associated genes (12,14). However, the pathogenesis remains
81 unknown in about 20-30% of the patients, pinpointing the need for identifying new
82 genes or mechanisms responsible for NS pathogenesis (5,15). Interestingly, we have
83 previously reported a double heterozygous patient carrying both an inherited *SOS1* and
84 a *de novo RAF1* variants (7). As the family carriers of that *SOS1* variant alone exhibited
85 a subclinical phenotype, we have hypothesized that the co-expression of additional
86 subclinical mutant effectors of the RAS pathway may have an addictive effect thus
87 contributing to the pathogenesis. By means of NGS target approach, we performed the
88 resequencing of RAS/MAPK pathway genes on eight patients with a clinical diagnosis
89 of NS and negative for the conventional NS mutation analysis. In three unrelated
90 patients, we have identified novel affecting function variants in the recently described
91 *LZTR1* NS-gene and in two new identified genes, *LRP1* and *RASAL3*, never associated
92 with NS. Furthermore, the co-occurrence of sub-clinical variants in two RAS-pathway
93 genes in the two probands prompted us to propose digenic inheritance as an alternative
94 pathogenic mechanism occurring in some NS patients, negative to conventional genetic
95 analysis.
96

97 **MATERIALS AND METHODS**

98 **Patients and cell samples**

99 Informed consents for genetic studies and publication of case reports were obtained
100 from all enrolled individuals or parents. Blood samples of these patients had been
101 collected in the laboratories of the University of Milan mainly from two hospitals,
102 Fondazione Policlinico San Matteo in Pavia and Istituto Auxologico Italiano in Milan

103 The treatment of human subjects complies with the Declaration of Helsinki, and the
104 Research Ethics Committee of IRCCS Istituto Auxologico Italiano (ID number
105 08C607_2006) approved the study. Epstein Barr Virus (EBV) immortalized PBMCs,
106 were provided by Galliera Genetic Bank from patients' lymphocytes (12)
107 (Supplementary File).
108
109 **Sequencing analysis and quantitative real-time PCR (qPCR)**
110 Constitutional DNA and total RNA were extracted from whole blood according to
111 standard procedures. PCR was carried out following standard procedures using specific
112 oligonucleotides (Table S1). The PCR products were sequenced and resolved on an
113 automated ABI-3130xl DNA genetic analyser (Thermo Fisher).
114 Total RNA (500 ng) was reverse transcribed using the iScriptTM cDNA Synthesis Kit
115 (Bio-Rad Laboratories Inc., Barkeley, CA, USA) and specific oligonucleotides allowed
116 to amplify *A2ML1* gene (Table S1), *TBP* gene was used as a housekeeping control
117 (Supplementary File).
118
119 **NGS analysis and protein modelling**
120 The enrichment of regions of interest was performed using Agilent SureSelect XT
121 technology. Target resequencing was performed on eight patients' samples by Illumina
122 MiSeq platform. Subsequently whole exome sequencing (WES) was performed on a
123 trio, as the proband was found uncharacterized in the previous analyses. For each
124 sample, a MiSeq run was performed. Raw reads of NGS data are available in NCBI
125 Short-read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/) under accession number
126 PRJNA607236.

127 The detailed bioinformatics analyses applied to NGS data are reported in
128 Supplementary File. To achieve multiple sequence alignment, necessary for protein
129 modelling, protein sequences were obtained from Uniprot and the CLUSTAL O servers
130 were employed. For protein modelling, multiple protein sequences were retrieved from
131 Uniprot and protein models were obtained through the Swissmodel Target-Template
132 Alignment module. The final alignments were carried out by means of ClustalO
133 application (Supplementary File).
134
135 **Array comparative genomic hybridization (aCGH) analysis**
136 The aCGH experiments were performed using a commercial SurePrint G3 ISCA
137 CGH+SNP Microarray Kit, 4x180K slides (Agilent Technologies Inc., Santa Clara, CA,
138 USA) in accordance with the manufacturer's instructions.
139
140 **Immunoblotting**
141 Proteins were extracted in 7M urea, 2M thiourea, 4%CHAPS, 30mM Tris, 1mM PMSF
142 (Sigma Aldrich), and 10µl/ml phosphatase inhibitor cocktails A and B (Santa Cruz
143 Biotechnology). Blots were incubated with anti-SAPK/JNK, anti-phospho SAPK/JNK
144 (Cell Signaling Technology), anti-ERK1/2 and anti-phosphoERK1/2 (ThermoFisher
145 Scientific) rabbit polyclonal antibodies (Supplementary File).
146
147 **Statistical analysis**
148 The ratio of phosphorylated over the total SAPK/JNK and ERK1/2 band intensity after
149 western blot was evaluated by applying the ANOVA with post-hoc Tukey HSD
150 (Honestly Significant Difference) tests ($n = 3$, $p\text{-value} < 0.01$ or $p\text{-value} < 0.05$). The

151 co-occurrence probability of the two *SOS1* and *LRP1* variants was calculated
152 considering their minor allele frequency (MAF) in both the whole 1000genomes dataset
153 and in the European subgroup (503 individuals). We also carried out the Fisher exact
154 test (http://vassarstats.net/odds2x2.html), and the Peto Odd Ratio (OR) was calculated
155 for the events of co-occurrence of the two variants in both our cohort of patients and in
156 the European subgroup of 1000genomes.
157

158 **RESULTS**

159 **Patients**

160 Eight patients (Pts 43, 49, 53, 55, 56, 62, 67, 69) out of a cohort of 60, negative after
161 conventional sanger sequence analysis, with or suggestive of NS phenotype, have been
162 molecularly characterized. The clinical features of the above patients are shown in
163 Table 1, with the exception of Pt 62, who has been meanwhile diagnosed to have
164 mosaicism for a small supernumerary marker chromosome derived from chromosome
165 19 and described by Recalcati et al. (16) as patient 1. Whenever possible, for each of the
166 clinical feature reported in Table 1 the corresponding item in the HPO (Human
167 Phenotype Ontology) phenotype vocabulary (17,18) has been indicated.
168 After the NGS target resequencing, the clinical phenotypes of the patients were
169 meticulously re-assessed by means of the same clinician. Patients 43, 53, 56 and 67
170 could be re-evaluated together with their family members, while for patients 49 and 55
171 only the clinical form filled in during the patients' enrollment could be revised. Patients
172 43, 53 and 56 have a clinical diagnosis of NS based on the criteria defined by van der
173 Burgt e al. (3) and exhibit the typical NS face dysmorphology. Similarly, Pt 69 could be
174 suggestive for NS diagnosis. Patient 67 instead had been candidate for screening in the

175 spectrum of RASopathies due to relative macrocephaly, hypotonia, poor growth and
176 growth hormone deficiency, congenital heart defect, and after the exclusion of the other
177 hypothesized clinical conditions. From their clinical records, it can be hypothesized that
178 Pt. 55, exhibiting hypoplastic supra-orbital ridges and blepharophimosis, may have a
179 different diagnosis from NS. Similarly, Pt 49 exhibited very few of the typical NS
180 craniofacial features and a CHD not specific to NS (Table 1). All of the parents and
181 siblings that could be reviewed (families of Pts 43, 53, 56 and 67, Figure 1) were
182 confirmed unaffected. Families' history was unremarkable with the exception of that of
183 Pt 56: he was the second born fourth child from Moroccan consanguineous parents, as
184 his paternal grandfather, II-3, and the mother, I-1, of his maternal grandfather, II-4,
185 were siblings (Figure 1d). The first pregnancy (IV-3) of his mother was reported to
186 interrupt at the seventh month for polyhydramnios; the second pregnancy (IV-4) was
187 reported to end at the fourth month of gestation without an apparent reason. The
188 relatives-clinical records were not available. Both parents were karyotyped to rule out
189 balanced chromosomal rearrangements. Moreover, the paternal cousin IV-2 was
190 reported to be affected by hypertrophic cardiomyopathy; the paternal cousin IV-1, who
191 was born from consanguineous parents too (III-1 and III-2 are cousins), by slight
192 intellectual disability. Unfortunately, their genotypes, although requested in Morocco,
193 were not carried out.
194
195 **NGS target resequencing**
196 To identify the affecting function variants associated with the clinical condition of the
197 enrolled patients, we designed a target sequencing panel including genes conventionally
198 associated with RASopathies, genes recently associated with NS and genes belonging to

199 the RAS/MAPK pathway and never associated with RASopathies, for a total of 26
200 genes (Table S2). We generated 2.9 million reads per sample on average. After
201 duplicate removal, we obtained an average of 2.8 million reads mapped on NCBI
202 human reference genome (build GRCh37). The mean depth was 747X (ranging from
203 384 to 899), with more than 99% of the targeted regions covered by NGS reads in each
204 sample (Table S3). Only 317 variants passed all the filtering steps imposed by our
205 pipeline (e.g. low-depth, strand-bias etc.) (Supplementary File). By means of Annovar,
206 the annotation analysis revealed 312 single-nucleotide polymorphisms (SNPs) and five
207 deletions. The majority of SNPs occur within intronic regions (114), while the
208 remaining are located in the 3′UTR or downstream (99), in 5′UTR or upstream (18), in
209 intergenic regions (20) and in exons (61). Among the coding variants, 16 and 45 are
210 missense and synonymous respectively (Table S4). Aiming at selecting coding non-
211 silent rare variants in 1000genomes database (1000g2015aug_eur), we applied a
212 stringent filter, according to a MAF<0,05, obtaining eight rare variants in five genes
213 (Table 2). To reduce false positive results, we applied a combinatorial approach by
214 comparing different prediction tools for assessing the possible effects of non-
215 synonymous SNPs (snSNPs) (Table S5). All the selected variants were validated by
216 Sanger sequencing. No affecting function variants were identified in patients 49, 55,
217 62 and 69. Chromosomal unbalances were ruled out by aCGH analysis. Proband 43
218 was found to be a carrier of the novel missense variant c.355T>C (p.(Tyr119His)) in
219 the exon 4 of *LZTR1* (Figure 1a, Table 2). This *de novo* variant was predicted
220 deleterious by the majority of *in silico* tools applied (Table S5). The novel variant
221 c.1430C>T (p.(Ala477Val)) in the exon 13 of *LZTR1*, was identified in heterozygosis
222 in Pt 53 (Table 2; II-2, Figure 1b) and was paternally inherited. The variant was

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223 never reported (Table 2) and was predicted damaging by different *in silico* tools
224 (Table S5). Moreover, three maternally inherited *LRP1* missense variants were
225 identified in heterozygosis: c.136C>T (p.(Arg46Trp)), c.5977C>T (p.(Arg1993Trp)),
226 c.8591G>A (p.(Arg2864His)) (Table 2; II-2, Figure 1b). This gene encodes a member
227 of the low-density lipoprotein receptor family of proteins, and interacts with FGF
228 receptors and CBL, mediating its ubiquitination activity involved in RAS pathway
229 regulation. All these variants were reported as deleterious, in particular the c.5977C>T
230 (p.(Arg1993Trp)) variant was predicted damaging by all considered tools (Table S5).
231 The patient 67 showed the paternally inherited variant c.650C>T (p.(Ala217Val)),
232 rs1800127 (Table 2; II-4, Figure 1c) in the exon 6 of *LRP1* predicted as tolerated (Table
233 S5). This patient also showed the maternally inherited *SOS1* variant c.1964C>T
234 (p.(Pro655Leu)), rs56219475, that was also carried by the unaffected sister II-3 (Figure
235 1c). The *in silico* predictions on the effect of this variant were discordant (Table S5), but
236 the variant was previously reported as benign (6). Given these results, we hypothesized
237 an addictive effect of the two variants that co-segregate in the proband. At this purpose,
238 we interrogated 2504 individuals included in the 1000genomes database (phase 3). The
239 two *LRP1* and S*OS1* variants display a frequency of 0.00698 and 0.00319 in the
240 1000genomes database, while in our cohort we detected 0.0199 and 0.0119,
241 respectively. Furthermore, we assessed if these two variants are never present in the
242 same individual. We used Fisher exact test to compare the frequency of co-occurrence
243 in the 1000 genomes EUR (N=503 individuals, no occurrences) and in our sample set
244 (N=8 patients, 1 co-occurrence), and this difference is statistically significant (p-
245 value=0.015, Peto OR = 5.50). Given the frequency of the two variants, the probability
246 of co-occurrence in our small sample set (8 patients) is as low as p-value=0.00189,

suggesting that this event should not be expected in the considered cohort, indicating that co-occurrence of *LRP1* and S*OS1* in our cohort with a frequency 0.125 is not a random event. Furthermore, to verify whether this event is due by chance, we evaluated in the 1000genomes database the co-occurrence of any two rare variants in all the genes included in our NGS panel. According to the previous analysis, we retained the non-synonymous variants with a MAF <0.05% and predicted to be damaging by at least two predictor tools. Following the identification of the individuals carrying more than two variants, we found that only 16 subjects, out of 2504 individuals (frequency 0.0064), shared the two variants *SOS2* c.1448G>A (p.(Ser483Asn)) and *A2ML1* c.1799T>C (p.(Val600Ala)). The novel missense variant c.2882A>G (p.(Asp961Gly)) in exon 24 of *A2ML1* (Table 2) was found in heterozygosis in patient 56 (Table 2; IV-6, Figure 1d). The variant was shared with both unaffected consanguineous parents (III-5 and III-6) and with the unaffected sister (IV-5). In addition, II-3 and II-4, who were reported to be healthy, are obligate carriers. All these findings suggest that c.2882A>G (p.(Asp961Gly)) is not sufficient to cause the phenotype (Figure 1d). We, then, investigated whether differential allelic expression (DAE) in whole blood as well as in epithelial tissue could be responsible for the incomplete penetrance. No significant difference of *A2ML1* specific alleles expression was observed comparing the proband to his sibling (*data not shown*).

Whole exome sequencing of trio 56

To elucidate the genetic bases of NS in family 56, we hypothesized the involvement of genes different from those belonging to the RAS pathway and we performed WES in the trio. At total of 49.11 million reads per sample was obtained; after duplicates

removal, and an average of 47.03 million reads were mapped on the NCBI human reference genome (build GRCh37). On average, the mean depth was 37X with more than 96% of the targeted regions covered by NGS reads in each sample (Table S6). All the selected variants were validated by Sanger sequencing. The condition of compound heterozygosis was detected for two variants in *RASAL3* gene, encoding a protein with pleckstrin homology (PH), C2, and Ras GTPase-activation protein (RasGAP) domains: the c.1963C>A (p.(Gly655Ser)) variant (rs199734851) in the exon 13 of *RASAL3* was paternally inherited, while the c.751C>G (p.(Leu251Val)) (rs58123634) in exon 7 of the same gene was maternally inherited (Table 2; Figure 1d). *In silico* predictions of the deleterious effect of these variants were reported in Table S5.

Protein modelling and *in silico* prediction of protein stability of deleterious nonsynonymous variants

Sequences of genes with substitutions in encoding regions were analyzed. In detail, the protein structure of A2ML1, LZTR1, RASAL3, SOS1 and LRP1 were modelled (see Supplementary File for details). The obtained model of A2ML1 allowed us to evaluate the c.2882A>G(p.(Asp961Gly)) identified variant. The residue substitution localizes in the protein core and is not directly involved in post translation modification (PTMs) or disulfide bridges. The 961 residue seems to have a role in proteases-binding or in their recognition and the substitution of a charged residue with a small apolar one may modify the protease binding activity. This substitution may be destabilizing because it is very close to the typical thiol ester sequence (969-973) (19) involved in the thiol ester bond (970-973) (Figure 2a). Interestingly, this

sequence is distinctive of the α-Macroglobulin, is involved in protein reactivity (19) and it is localized on the opposite side of the bait domain on a large binding cavity; the bait domain is generally cleaved by proteases, in order to induce a conformational change and entrap the protease itself (19).

The LZTR1 model allowed us the evaluation of the two identified variants (Table S7): the substitution c.355T>C (p.(Tyr119His)) (Figure 2b) that is predicted to destabilize the protein structure while the substitution c.1430C>T (p.(Ala477Val)) is located outside the modelled protein (ending in position 325) portion and is predicted neutral while (Table S7). Noticeably, His119 in its neutral form can establish π-π stacking interactions with the neighbor His120, therefore possibly reducing the flexibility of His121, which interestingly is a catalytic residue.

Concerning to RASAL3, all the three stability prediction tools reported c.1963G>A(p.(Gly655Ser)) variant as destabilizing for the protein structure (Table S7). The substituted residue localizes on the protein surface (Figure 2c), within a loop, at the end of the functional RAS-GAP domain and therefore the substitution could be involved in the binding interface with RAS destabilizing this docking.

Considering the variant identified in SOS1 (Table S7), we assessed that the substitution c.1964C>T (p.(Pro655Leu)) is localized on the protein surface, in a loop, within the RAS-GEF domain (Figure 2d). Since Proline residues are highly functional for protein structure also in loop conformations, this variant may be destabilizing for the protein structure.

The new candidate NS *LRP1* gene, it encodes a large protein of about 4500 residues, mainly composed by LDL-receptor repeated domains (20) and by EGF-like domains, not equally distributed along the sequence (Figure 3e). Due to the protein length, we

were not able to obtain a single model for the whole structure, but we obtained four models not super-posable including residues 1 to 3332 (Figure 3 a-d) (Supplementary File for details). All of the four identified substitutions of LRP1- c.136C>T (p.(Arg46Trp)), c.650C>T (p.(Ala217Val)), c.5977C>T (p.(Arg1993Trp)) and c.8591G>A (p.(Arg2864His)) - were predicted to be destabilizing for the protein structure as indicated by negative difference of free energies of wild type and mutant structures (Table S7) and were modelled. The first substitution, c.136C>T (p.(Arg46Trp)) (Figure 3a), is localized in a partially folded region and this substitution may influence cysteine 65, is involved in a disulfide bridge with Cys48. The substitution c.650C>T (p.(Ala217Val)) (Figure 3b) is conservative and localizes on the receptor surface, therefore the involvement in the NS pathogenesis cannot be ascribable to protein structure destabilization. The substitution c.5977C>T (p.(Arg1993Trp)) (Figure 3c) displays an increased steric hindrance in a packed region at the internal side of the YWTD-beta propeller. Moreover, substituting a positively charged residue with an aromatic nonpolar aminoacid destroys the high number of hydrogen bonds and salt interactions, which involve Arginine. This condition induces higher flexibility of the involved protein portion. Furthermore, a larger sidechain may have effects on the glycosylation of residue Asp1995, necessary for protein structure. The variant c.8591G>A (p.(Arg2864His)) (Figure 3d) is localized in a disordered region, but in the nearby of a YWTD-beta propeller. This type of substitution may be classified as not destabilizing because a positively charged residue is substituted with another positively charged residue, but with a lower isoelectric point. However, the variant may influence the disulfide bridge of residue 2865 with Cys 2884.

343

344 **Extracellular signal regulated kinase (ERK) and Stress-activation protein**
345 **kinase/Jun-amino-terminal kinase (SAPK/JNK) in patients 53 and 67**

346 We carried out specific assays to verify the activation of both ERK1/2 and
347 SAPK/JNK, thanks to the availability of immortalized lymphoblastoid cell lines
348 obtained from the PBMCs of both trios 53 and 67, showing a co-segregation of more
349 hypomorphic variants. In fact, the evaluation of ERK1/2 phosphorylation increase is
350 a common assay to validate a new NS variant, but more recently a *SOS1* variant has
351 been observed to activate SAPK/JNK instead of ERK in an NS patient (7). While the
352 levels of ERK1/2 phosphorylation, assessed by immunoblotting, were not
353 significantly different in patients 53 and 67 compared to their healthy parents and
354 unrelated controls (Figure 4), SAPK/JNK was activated in both NS patients. The
355 ratio of the phosphorylated over the total SAPK/JNK band intensities was calculated
356 for each sample. As expected, the anti-SAPK/JNK antibody revealed two bands at
357 ~46 (p46) and ~54 KDa (p54), respectively (Figure 5a-b). Of them, only p54 resulted
358 phosphorylated in both patients (Figure 5-d). A statistically significant increment
359 (ANOVA + Tukey, $n = 3$, $p\text{-value} < 0.01$) of phospho-SAPK/JNK was detected in
360 sample 67 compared to 67M, 67P and unrelated control (Figure 5e) and in sample 53
361 compared to 53M, 53P and unrelated control (Figure 5f). In addition, for sample 53, a
362 second band, p54B, was detected both in total and in phosphorylated SAPK/JNK
363 blots (Figure 5b).

364

365 **DISCUSSION**

366 Molecular diagnosis remains unknown at least in 20-30% of NS patients (5),
367 therefore new genes and genetic mechanisms are expected to play a role in the
368 disease. NGS approach led to the identification of new NS/NS-like genes (4,5,21), all
369 encoding members of RAS/MAPK pathway. Most of them have been identified in
370 less than 10 individuals (4), suggesting that all RAS/MAPK genes should be
371 investigated as potentially implicated in the pathogenesis of NS, and more in general
372 of RASopathies. Accordingly, we designed an extensive NGS target resequencing
373 panel. A novel *de novo* missense variant c.355T>C (p.(Tyr119His)) was identified in
374 *LZTR1* in proband 43, who exhibits a typical NS phenotype with mild severity, since
375 her development, intellectual skills and growth were unremarkable (Table 1). *LZTR1*
376 encodes a protein member of the BTB-kelch superfamily. Recently, a significant
377 amount of data referring to functional role of *LZTR1* in RAS signaling and the impact
378 of these variants on protein function, have been reported (8,22). Yamamoto et al.
379 identified rare variants of *LZTR1* among which (p.(Tyr119Cys)) was reported as a *de*
380 *novo* event occurring with NS phenotype (23). As the substitution here described is
381 not conservative, involves the same aminoacidic residue previously described (23)
382 and may affect a nearby catalytic residue, we propose it as a new pathogenic NS-
383 associated variant. Another novel variant in *LZTR1* (NM_006767.3): c.1430C>T,
384 predicted to be deleterious, but inherited from an unaffected father, was identified in
385 proband 53. This variant could not be modelled and was not associated with NS
386 phenotype if inherited alone. Nonetheless, in proband 53, we observed that the co-
387 occurrence of *LZTR1* (NM_006767.3): c.1430C>T variant~~s~~ with the three *LRP1*
388 variants NM_002332.2: c.[136C>T;5977C>T;8591G>A], all inherited from the
389 unaffected mother (Figure 1b), segregates with NS phenotype. This complex

390 condition suggests a digenic inheritance model. LRP1 is involved in intracellular
391 signaling functions as well as in lipid metabolism. It is a physiological modulator of
392 the platelet-derived growth factor (PDGF) signaling pathway and can directly interact
393 with the ubiquitin E3-ligase CBL, already associated with NS (24,25). This evidence
394 suggests a Sprouty-like role for LRP1 in modulating ubiquitination and endocytosis
395 of the tyrosine-kinase (Trk) receptors such as PDGFRbeta (26,27). Moreover, LRP1
396 regulates the A2ML1 activity by promoting its internalization, thus maintaining its
397 clearance (28). Interestingly, also the proband 67 paternally inherited the *LRP1*
398 (NM_002332.2): c.650C>T variant, which was not associated with NS phenotype if
399 inherited alone. In addition, the proband 67 also maternally inherited the *SOS1*
400 (NM_005633.3): c.1964C>T missense variant (Figure 1c). This variant is not rare in
401 the population and has been previously reported as benign (6), however the Proline
402 residue is highly functional for the protein structure, therefore the substitution could be
403 destabilizing for the structure. All the identified *LRP1* variants do not seem to be
404 sufficient to determine the manifestation of NS phenotype, as we observed in both
405 cases the co-occurrence with *LZTR1* (Pt. 53) and *SOS1* variants (Pt. 67). While the
406 *LRP1* and *SOS1* variants detected in patient 67 are singularly reported in genomes
407 from 1000genomes data set, interestingly, their co-presence does not occur. Both the
408 calculation of co-occurrence probability and the application of Fisher exact test and
409 OR, indicate that the co-presence of the identified subclinical *LRP1* and *SOS1*
410 variants is expected as a very rare event. The identification of this condition in one
411 patient of a cohort of eight individuals (frequency 0.125), together with the very low
412 frequency of the co-occurrence of two any rare variants in the 1000genomes
413 population (frequency 0.0064), is consistent with its pathogenic significance,

414 invoking the hypothesis of a digenic model in NS. Digenic inheritance of subclinical
415 variants could partially account for pathogenic causes in 30% of diagnosed NS
416 patients, explaining the lack of identification of affecting function variants in this NS
417 subgroup (5). This hypothesis is consistent with the increase of phospho-SAPK/JNK
418 in the PBMCs cell lines of both patients in comparison to those of their parents, who
419 carried only one of the two mutated genes present in the probands. We observed no
420 significant changes in ERK1/2 phosphorylation, which is the functional marker
421 commonly considered to validate NS variants (5). Nevertheless, Longoni and
422 colleagues reported on the increasing of phospho-SAPK/JNK after overexpression of
423 SOS1 R497Q variant detected in the father and grandfather of a double heterozygous
424 NS patient, carrying both SOS1 and RAF1 variants (7), where both proband's
425 relatives showed subclinical NS traits. It is, therefore, possible that SAPK/JNK
426 pathway, which shares some common effectors with ERK1/2 pathway, might also be
427 involved in NS pathogenesis. We speculate that the co-occurrence of variants in both
428 *LRP1* and in *SOS1* or *LZTR1* may alter RAS pathway upstream causing
429 hyperactivation, likely by an addictive effect, of either ERK1/2 or SAPK/JNK in a
430 specific tissue fashion (29).
431 Patient 56 shared the *A2ML1* (NM_144670.5): c.2882A>G variant with all the
432 unaffected family members. Segregation of the variant in the family suggests that it
433 may be not associated with NS, nevertheless, our model predicted a possible effect
434 on A2ML1 protein function. Indeed, family member IV-1 (Figure 1d), who is
435 affected by slight intellectual disability, may be homozygous for the above variant in
436 *A2ML1*. Because A2ML1 is a protease inhibitor and binds to LRP1, regulating
437 activation of the MAPK/ ERK cascade, as in the case of LRP1, we hypothesized that

438 also this variant in *A2ML1* could contribute to alter the physiological modulation of
439 RAS/MAPK pathway and display NS insurgence in association with variants
440 occurring in other genes. At this purpose we performed WES analysis in the trio and
441 found that Pt.56 was a compound heterozygote for *RASAL3* (NM_022904.2):
442 c.[1963C>A]; and c.[751C>G] variants, which were maternally and paternally
443 inherited, respectively. The finding, as well as a low probability of loss-of-function
444 intolerance (pLI = 0.00) (30), is suggestive for *RASAL3* being involved in NS
445 pathogenesis according to an autosomal recessive mechanism. Indeed, the experience
446 of polyhydramnios in the third trimester during the first pregnancy (IV-3, Figure 1d)
447 of Pt.56's mother is highly suggestive of NS and of the likely existence of compound
448 heterozygosity for *RASAL3* pathogenic variants at least in IV-3 aborted fetus.
449 Noteworthy, Pt.56, who exhibited the typical NS gestalt and had a history of
450 polyhydramnios and hydrops fetalis during pregnancy, showed developmental delay,
451 severe speech and language delay associated with sensorineural deafness, and
452 moderate intellectual disability (Table 1). RASAL3 is a Ras GTPase-activating
453 protein, i.e. RasGAP (31,32) still little characterized. For instance, NKT cells from liver
454 of RASAL3-deficient mouse treated with α-GalCer have been shown to increase Erk
455 phosphorylation, suggesting that RASAL3 negatively regulates Ras/Erk signaling
456 (31,32). Despite this evidence may be encouraging about our finding, we believe that it
457 should have been fundamental to investigate how a predicted loss of function of a
458 RasGAP predominantly expressed in cells of hematopoietic lineages (31,32) can result
459 in a severely/fully expressed NS RASopathy. Unfortunately, PBMCs samples of
460 family 56 were not available, therefore it was not possible to investigate ERK1/2 or
461 SAPK/JNK phosphorylation levels. Furthermore, *RASAL3* may also perform a Ras-

462 independent function in neuronal signal transduction. Indeed, despite in the literature
463 *RASAL3* expression is studied in the lymphoid cells, according to BrainSpan
464 (https://www.brainspan.org/) and GTEx (https://www.gtexportal.org/home/)
465 databases, the gene is brain expressed prenatally (average level of expression=0.186)
466 and in infancy (average level of expression=0.208). Moreover, it keeps on being
467 expressed, at a higher level, during adult life (average level of expression=2375.5)
468 (33). Further functional studies will be needed to confirm our findings. Here we
469 provide a first evidence that digenic inheritance may be an alternative mechanism
470 involved in the etiopathogenesis of NS and RASopathies, as already hypothesized
471 (7,34). Moreover, we have identified two novel genes, *LRP1* and *RASAL3,* possibly
472 involved in the etiopathogenesis of NS/RASopathies. In particular, *RASAL3*, whose
473 affecting function variants have been identified in a family from a population with a
474 high rate of inbreeding, represents, after *LZTR1*, the second gene associated with
475 autosomal recessive inheritance in NS so far reported (13). Here we have identified
476 the presence of variants in major genes of the RAS/MAPK pathways in a subgroup of
477 NS patients selected from a cohort 60 with heterogonous NS phenotypes, but
478 negative for conventional NS mutation analysis. To gain more confidence in the
479 proposed alternative hereditary mechanisms, more NS patients and their families
480 should be investigated by means of NGS approach. This might be instrumental for
481 the discovery of subclinical variants in functionally related genes that are apparently
482 benign if inherited alone, but potential pathogenic if co-segregating in a single
483 patient.
484 In conclusion, our results together with the growing body of literature, indicate that
485 the routinely application of massive sequencing techniques is uncovering the

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486 emerging contribution of hypomorphic variants to the clinical phenotype, unlike the
487 application of Sanger method that led to interrupt the analysis when an affecting
488 function variant was detected. Geneticists need to be aware of the complexity of the
489 genetic characterization and counselling about NS and RASopathies. Therefore, the
490 revaluation of many monogenic Mendelian conditions characterized by a broad
491 spectrum of phenotypes, such as NS, may lead to the identification of different
492 inheritance models, able to explain variable expressivity and incomplete penetrance.
493

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660
661

662 **FIGURE LEGENDS**

663 **Figure 1: Pedigrees of NS patients and segregation analysis of the identified**
664 **variants.** Filled symbols, subjects with NS/NS spectrum; light-dotted filled symbols,
665 reported to be affected by slight Intellectual disability (d, IV-1) or hypertrophic
666 cardiomyopathy (d, IV-2); asterisk, members phenotyped and genotyped in the study;
667 Pt, patient; SA, spontaneous abortion.

668

669 **Figure 2: Structural predictions of A2ML1, LZTR1, RASL3, and SOS1.** A2ML1
670 structural prediction, substituted residue, Asp961Gly, is in blue stick and functional
671 regions are in evidence, thiol ester sequence in pink and bait region in red (a). LZTR1
672 model and focus on Tyr199His substitution, in grey wild type (wt) residue and in green
673 the model of Tyr in position 119 (b). RASAL3 model and focus on Gly655Ser
674 substitution, in grey wt residue and in white Ser in position 655 (c). SOS1 crystal
675 structure and focus on Pro655Leu substitution, in grey wt residue and in light-blue
676 Proline in position 655 (d).

677

678 **Figure 3: Assembly of LRP1 models and focus on LRP1 variants.** Each colour
679 identifies a partial model, starting and ending residue of each model is labelled. In the
680 focus the detail of Arg46Trp substitution, in red wild type (wt) Arg and in white Trp
681 variant (a), of Ala217Val, in red wt Ala and in white Val variant (b), of Arg1993Trp, in
682 orange wt Arg and in white Trp variant (c) and of Arg2864His, in blue wt Arg and in
683 white His variant (d). The domains organization is displayed (e), green pentagon
684 represents EGF domains, green rhombus represents LDL-receptor class A domain,
685 while in pink are represented YWTD domains.

686 **Figure 4: Activation ERK1/2 assay.** Representative immunoblot images of stress-
687 activated protein kinase (ERK1/2) (a), and phosphorylated ERK1/2 (b). Histograms
688 show the ratio of phosphorylated over the total ERK1 (p44) band intensity (O.D. =
689 optical density, mean ± SD) in patient 53 and 67 compared to their parents and
690 unrelated controls 524 and 548 (c). Histograms show the ratio of phosphorylated over
691 the total ERK2 (p42) band intensity (O.D. = optical density, mean ± SD) in patient 53
692 and 67 compared to their parents and unrelated controls 524 and 548 (d).
693 Phosphorylation levels were not significantly different in patients 53 and 67 compared
694 to their healthy parents and controls, applying the ANOVA + Tukey HSD tests ($n = 3$,
695 significance threshold: $p – value < 0.05$).
696
697 **Figure 5: Activation SAPK/JNK assay.** Representative immunoblot images of stress-
698 activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) (a, b), and
699 phosphorylated SAPK/JNK (c, d). Histograms show the ratio of phosphorylated over
700 the total SAPK/JNK band intensity (O.D. = optical density, mean ± SD) in patient 67
701 (e) and 53 (f) compared to their parents and unrelated controls 524 (e) and 548 (f).
702 Significant differences are computed applying the ANOVA + Tukey HSD tests ($n = 3$,
703 significance threshold: plain lines = $p\text{-value} < 0.01$, dotted lines = $p – value < 0.05$).
704
705