Cardiac defects, morbidity and mortality in patients affected by RASopathies. CARNET study Results

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KEY WORDS:

Noonan Syndrome, RASopathies, congenital heart defect; children; hypertrophic cardiomyopathy

ABSTRACT

BACKGROUND: Rasopathies are developmental disease caused by mutations in genes encoding for signal transducers of the RAS-MAPK cascade. The aim of the present study was to provide a comprehensive description of morbidity and mortality in patients with molecularly confirmed Rasopathy.

METHODS: A multicentric, observational, retrospective study was conducted in seven European cardiac centres participating to the CArdiac Rasopathy NETwork (CARNET). Clinical records of 371 patients with confirmed molecular diagnosis of RASopathy were reviewed. Mortality was described as crude mortality, cumulative survival and restricted estimated mean survival. Multivariable regression analysis was used to assess the impact of mutated genes on number of interventions and overall prognosis.

RESULTS: Cardiac defects occurred in 80.3% of cases, almost half of them underwent at least one intervention. Overall, 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 follow-up was 19.6 years. Ten patients died (2.7% of the entire cohort; 3.4% of patients with cardiac defect). Patients with hypertrophic cardiomyopathy (HCM) and age < 2 years or young adults, as well as subjects with biventricular obstruction and *PTPN11* mutations had a higher risk of cardiac death.

CONCLUSIONS: The risk of intervention was higher in individuals with Noonan syndrome and pulmonary stenosis carrying *PTPN11* mutations. Overall, mortality was relatively low, even though the specific association between HCM, biventricular outflow tract obstructions and *PTPN11* mutations appeared to be associated with early mortality, including immediate post-operative events and sudden death.

1. INTRODUCTION

RASopathies are a group of disorders, caused by mutations in genes encoding for signal transducers of the RAS-MAPK cascade, affecting 1 in 1000-2500 children [1-4]. Among these disorders, Noonan syndrome (NS), NS with multiple lentigines (NSML, also known with the acronym LEOPARD syndrome), Costello syndrome (CS), and cardiofaciocutaneus syndrome (CFCS) share a similar systemic phenotype, with a wide spectrum of congenital heart disease (CHD) and hypertrophic cardiomyopathy (HCM) as major associated features [5-10]. Genotype-phenotype correlations have been established, including pulmonary stenosis (PS) and *PTPN11* mutations, HCM and *RAF1*, *HRAS* and a subset of *PTPN11* mutations, and mitral valve defects (MVD) and *SHOC2* c.4A>G change [11-15].

To date, limited information is 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.

2. METHODS

2.1 Study design and population

This is a multi-centric, retrospective, observational study conducted in seven cardiac centres (Italy and UK) with expertise in RASopathies and participating in the CArdiac Rasopathy NETwork (CARNET). We retrospectively analysed clinical records of all patients with molecularly confirmed diagnosis of NS, NSML, CS or CFCS, followed up until July, 2014. Molecular diagnosis was performed through a combination of Sanger sequencing and targeted resequencing directed to scan the entire coding sequence of *CBL*, *PTPN11*, *SOS1*, *KRAS*, *HRAS*, *NRAS*, *SHOC2*, *RAF1*, *BRAF*, *MAP2K1* and *MAP2K2* genes, which had been recognized as RASopathy disease genes at the time of this study [1].

The following data were collected: date of birth and sex; clinical diagnosis and mutation; clinical phenotype; type of heart defect, including PS, atrioventricular canal defects (AVC), HCM, and other less common defects, such as atrial septal defects (ASD) and mitral and aortic valve diseases (MVD and AVD, respectively); timing and details of all cardiac procedures; date and cause of death, when appropriate. Data were centralized in a unique database. The study protocol was approved by the Bambino Gesù Children's Hospital's Ethical Committee (Protocol Number 605.13) and by the Ethical Committees of all the other participating centres.

3. STATISTICAL ANALYSIS

Data were presented as median and range, mean ± standard deviation (SD), or percentages and CI, as appropriate. Mortality was described as crude mortality.

Cardiac defects taken into account in the analyses were HCM, PS, and AVC; the other cardiac defects were grouped in the category "other heart defects" (OHD) and investigated only descriptively.

Similarly, in the descriptive analysis, rarer mutations with low recurrence in the cohort (i.e. *CBL*, *HRAS*, *KRAS*, *MAP2K1*, *MAP2K2*, *NRAS*, and *SHOC2*) were reported as "other genes" (OG), but were considered as single genes in the regression analyses.

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, we studied:

- the association between mutated genes and number of procedures received by each patient with a cardiac defect (interpreted as a count variable), adjusting for sex, presence of PS, HCM, AVC or OHD, and natural logarithm of years of follow-up

- the association of each syndrome with number of interventions, adjusting for sex, type of heart defect and natural logarithm of years of follow-up;

- the association between presence of PS, HCM, AVC and OHD, and the number of interventions, correcting for sex, gene, and natural logarithm of years of follow-up.

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 defect; 95% confidence intervals (95% CI) are shown for the cumulative survival KM plots.

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.

The effect sizes of the regression models are reported as adjusted incidence rate ratios (aIRR) for Poisson regressions, and as adjusted hazard ratios (aHR) for Cox regressions.

Poisson regression estimates were regularized using a Bayesian approach (*Data In*

Brief, table and statistical analysis). Bootstrap analysis was used to generate bias-corrected accelerated bootstrap confidence intervals (BCA CI 95%) and standardized effect sizes (sES) for all regression coefficients.

KM models were also used to describe mortality as cumulative survival at 1, 5, 10 and 20 years, as restricted estimated mean survival (on a 20-year follow up) and as function of mutated gene, clinical syndrome and specific cardiac defect.

KM plots are used to visualize KM models; forest plots reporting effect sizes and BCA CI 95% are presented for each regression analysis. In the plots, only the main genes (*BRAF, PTPN11, RAF1* and *SOS1*) were considered.

The software R ver. 3.2.0 was used for analysis and plotting. See *Data In Brief,* for a more complete description of every analyses and of the statistical techniques used.

4. RESULTS

4.1 Study population and heart defects

We enrolled 371 individuals with a RASopathy, 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). The date of birth was missing for 2 patients and the date of the last follow-up was missing for 33 subjects. Table 1 reports demographic, clinical and molecular characteristics of included patients. *Data in Brief, Table 1* shows the distribution of the affected genes by syndrome. Of note, one patient, carrying a missense mutation in *HRAS* mutation (p.Gly13Asp) rarely occurring in CS, was diagnosed as affected by NS based on his clinical features, in line with a recent report [16]. This patient was not affected by heart disease.

Two hundred ninety-eight patients (80.3%) had a cardiac involvement, either with a single anomaly or multiple defect(s). More than a half subjects had PS (59%), mostly isolated (81% of all patients with PS). Eighty-one (27%) had HCM; among these, 32% also had MVD 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 (Table 1).

4.2 Morbidity

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 (PBPV)). In 7 patients

(12%), a significant pleural or pericardial effusion was the reason for an early reintervention (*Data in brief, Figure 1*).

Genes

We analysed the association between each mutated gene and number of interventions (adjusting for sex, cardiac defect and length of follow up), including both interventional and surgical procedures (*Data in Brief, Table A and Figure 2A*). 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])

According to the KM curves (Figure 1A), *PTPN11* mutations increased the risk of early intervention, the curves 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. However, the Cox analysis did not show a significant effect on hazard of intervention for any of the disease genes, if the effect of the cardiac defect was taken into account (*Data In Brief, Table B and Figure 2B*).

Syndromes

Poisson regression (adjusting for sex, type of cardiac defect and length of follow up), showed that subjects with CFCS had a significantly lower number of interventions (aIRR 0.264, BCa95% CI: [0.0767, 0.909]), while NS was associated with a significantly higher number of interventions compared to other syndromes (aIRR 1.88, BCa95% CI: [1.19, 3.17]) (*Data In Brief, Table C and Figure 2C*).

Kaplan-Meier curves showed that NS was associated with a persistently higher risk of intervention (Figure 1B); CFCS patients had a lower risk of intervention, although the population was very small. Of note, NSML seemed to be positively associated with an

overall reduced cumulative risk of intervention, although this effect tended to decrease towards adulthood. CS population was too small to draw conclusions.

Cox analysis confirmed that NS was associated with a higher cumulative risk of interventions, independently from the type heart defect (aHR 2.13, BCa95% CI: [1.12, 3.75]) (*Data In Brief, Table D and Figure 2D*).

Heart defects

Subjects with PS (aIRR 2.55, BCa95% CI: [1.59, 3.9]) and AVC (aIRR 3.86, BCa95% CI:[2.37, 6.08]) had a significantly higher number of interventions (when adjusting for sex, mutated gene, cardiac defect and length of follow up, *Data In Brief, Table E and Figure 2E*). Kaplan-Meier curves showed that PS and AVC had a consistently higher risk of earlier intervention (Figures 1C and 1D) and HCM had a reduced risk of intervention compared to patients affected by other defects. In patients with HCM, the lower risk of intervention disappeared at around 15-20 year of age (Figure 1E). Cox analysis (*Data in Brief, Table F and Figure 2F*) confirmed the risk of an earlier intervention for PS (aHR 2.93, BCa95%CI: [1.47, 5.36]) and AVC (aHR 6.12, BCa95% CI: [2.62, 13.6]).

4.3 Mortality

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 (Figure 2A). Kaplan-Meier curves for risk of mortality are reported by gene (Figure 2B), syndrome (Figure 2C) and type of heart defect (Figure 2D, 2E, 2F).

Ten patients died (2.7% of the entire cohort; 3.4% of patients with cardiac defect). Six subjects died during the first 2 years of life. Among them, two patients died from leukaemia. 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. Specifically, two patients, both affected by HCM and heterozygous for a *PTPN11* mutation, died before 2 years of age from sudden death, before receiving any surgical treatment. Of these, one was affected with NS and had a severe subaortic obstruction associated with the HCM, while the other patient was affected with NSML and had a biventricular obstruction due to concomitant muscular subaortic and severe pulmonary valve stenosis. The remaining six patients underwent at least one procedure. Of these, two patients died for late complications during the post-operative follow up. Both these patients were affected by HCM and had a *PTPN11* mutation. One subject (NS) died from rejection eight months after heart transplantation. The other patient (NSML), with a biventricular outflow tract obstruction, underwent multiple surgical treatment for a pulmonary stenosis, and died from sudden death more than 5 years after surgery. The remaining 4 patients died from immediate post-operative complications within 30 days from the intervention. One patient (NS, *PTPN11*), affected with partial AVC and aortic valve stenosis, died from a cardiac tamponade after a second intervention in which he received aortic root replacement with coronary reimplantation. He had previously undergone aortic valvulotomy and AVC correction as first intervention. One patient who was born preterm (34 weeks of gestational age; NS, *PTPN11* mutation) and had with severe pulmonary stenosis and severe aortic stenosis, died from low cardiac output following a surgical pulmonary valvulotomy. He had previously received an unsuccessful balloon pulmonary dilation. Finally, two patients, one with NS-like features (so called Mazzanti syndrome due to the recurrent c.4A>G mutation in *SHOC2*) [15], and one with NSML, associated with a mutated *PTPN11* allele, both affected with HCM and severe left and right outflow tract obstruction, died from low cardiac output following a surgical intervention aimed at reducing biventricular outflow obstructions. MVD, AVD and biventricular obstruction were frequent in the group of patients who died, particularly in the subgroup of patients who died for

immediate post-surgical complications. Table 2 shows molecular and clinical details for each patient who died.

5. DISCUSSION

In this study, we retrospectively analysed a large cohort of patients affected by RASopathies with a confirmed molecular diagnosis. Heart involvement, morbidity and mortality were analysed taking into account both the involved disease genes and the clinical diagnosis. Our results 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.

5.1 Prevalence and clinical significance of heart defects

As expected since the relatively higher proportion of subjects with NS, PS was the most common heart defect in our study population, followed by HCM and ASD. These results are consistent with those reported in a recent study by Prendiville [17] (which however included only patients with NS) and by Lin et al., who found a high prevalence of PS and HCM in a population including NS, NSML, CS and CFCS [18]. Consistently with previous reports, MVD and AVD also recurred as associated features in NS and NSML patients [19,20], as isolated defects or as part of the AVC or obstructive HCM spectrum. When associated with AVC or HCM, AVD and MVD represent a marker of phenotypic complexity and severity, deserving a comprehensive diagnostic evaluation and careful consideration for a tailored surgical approach [18-22].

5.2 Morbidity

Among patients affected with a heart disease, almost half underwent a percutaneous and/or surgical intervention. A second intervention was frequent (more than 40% of patients that underwent a first intervention).

As expected, individuals with AVC were those who most frequently required an early intervention, followed by NS patients with PS and *PTPN11* mutations. As shown by the KM curves, patients affected by PS and NS displayed a need for a surgical/percutaneous intervention throughout the entire time of follow-up. In the subgroup of patients who underwent a second procedure, the main cause for reintervention was recurrence of PS (60% of these patient), mainly for an unsuccessful PBPV. 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 [17]. Similarly, McCrindle et al. reported a significant need for reintervention in patients with PS after PBPV, due to the specific morphology of the pulmonary valve in these patients, characterized by dysplastic pulmonary leaflets [23].

Regarding patients with HCM, our study suggests that the risk of intervention in these patients is lower after the first years and before 15-20 years of age, in line with Prendiville et al. and Poterucha et al. [17,24]. 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 [17,24]. We reported MVD as a marker of clinical complexity in HCM patients. Maron et al. recently reported that the length of the mitral valve leaflet is significantly increased in HCM patients compared with age, sex and body size-matched controls without cardiovascular involvement [25]; structural mitral valve abnormalities should be regarded as a primary phenotype of HCM also in patients with NS [19]. These characteristics may help to explain the pathophysiology of the subaortic gradient in patients with obstructive HCM with mild septal hypertrophy and its potential role in surgical and/or morbidity outcome.

Subaortic stenosis due to accessory fibrous connective tissue and/or anomalous insertion of the mitral valve or anomalous left ventricle papillary muscle, should be carefully

taken into account regarding morbidity and mortality, also in patients with AVC, particularly in RASopathies [19-21,26]. We noticed that the surgical approach aimed at relieving left and right ventricular stenosis could be very difficult or unsuccessful in infants and neonates with severe HCM consisting in biventricular outflow tract obstruction.

In our 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.

Finally, a significant cause of reintervention (more than 10%) was post-procedural pericardial or pleural effusion. Our results are in agreement with previous findings in patients with RASopathies [17,27]. The higher risk of pericardial or pleural effusion may be due to specific lymphatic anomalies reported in patients affected with these syndromes.

5.3 Mortality

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. Of note, 80% of patients died for a specific cardiac cause, *e.g.* post-operative low cardiac output, sudden death or heart transplant rejection. 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 (of these, 3 died before 2 years of age).

Seven patients in the group of patients who died received at least one intervention. Five of them underwent a further procedure. Immediate post-operative complications were

cause of death in 4/7 cases. Interestingly, all these patients had an associated MVD/AVD, suggesting the possible role of these additional cardiac malformations in worsening the clinical expression the heart defects [19,21,25,26]. In the subgroup of patient who died for cardiac causes, biventricular outflow tract obstruction was frequent (5/8 patients), in association with HCM or CHD, and can therefore be considered as a surgical risk factor.

When analysing the age distribution of the patients who died, we found that the majority of deaths occurred in the first two years of life, or among young adults ($\geq$ 20 years of age). Prendiville et al. reported a mortality of 15% in patients with NS and HCM [17], while mortality of patients with RASopathies and HCM was 9% in this survey. Data from the Pediatric Cardiomyopathy Registry showed that crude mortality was worse in syndromic HCM (mainly, NS) compared to non-syndromic HCM [28,29]. Specifically, Colan et al. showed that children with syndromic HCM and with an early onset HCM phenotype (<1 year of age) had a poor prognosis, with a 5-year survival of 65.8% [28]. Wilkinson et al. showed the importance of heart failure at HCM onset as a risk marker of prognosis, with a one-year survival in 31% of patients [29]. Limongelli et al. suggested that patients with NSML and HCM require a periodic and careful cardiac risk assessment due to the frequent occurrence of life-threatening arrhythmias and sudden death [22].

6. STUDY LIMITATIONS

The retrospective design of the study represents as *per se* a limitation. Recently discovered genes, e.g. *RIT1, SOS2* and *LZTR1* [30,31], have not been tested in our cohort, therefore excluding from our population RASopathy patients with negative genetic characterization may have biased our results. Another limitation concerns the statistical analysis. Some variables (e.g. patients with *RAF1* gene mutation, CS syndrome, those who received more than two reinterventions, deceased patients), had a very low numerosity, and this could have impaired the precision of the analysis. However, using the bootstrap and a Bayes-based regularization, we increased the sensitivity of the analysis.

7. CONCLUSIONS

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. Cardiovascular involvement is a common feature of these disorders. 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 (particularly, if biventricular obstruction coexists), *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.

Defining “red flags” for a more severe cardiac phenotype spectrum will be essential to target specific treatment phenotype/gene/mutation-specific in patient affected with RASopathies.

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FIGURE LEGEND

Figure 1. Kaplan-Meier curves reporting changes in number of years free of surgical intervention by gene (A), syndrome (B) and specific heart defects (C to E).

Figure 2: Kaplan-Meier curves reporting estimated years of survival, overall (A) and by gene (B), syndrome (C) and specific heart defects (D to F).