UNIVERSITÀ DEGLI STUDI DI GENOVA

Scuola di Scienze Mediche e Farmaceutiche

Dottorato di Ricerca in Scienze Pediatriche

Curriculum Specialità Pediatriche

Somatic *NF1* loss of heterozygosity associated with NF1-related pectus excavatum deformity: a new insight in pathogenesis?

Mutazione somatica con perdita dell'eterozigosi di deformità toracica in paziente affetto da Neurofibromatosi di tipo 1: una nuova visione della patogenesi?

Relatore:
Chiar.mo Prof. Pasquale STRIANO

Correlatore:
Dott.ssa Maria Cristina DIANA

Candidato:
Dott.ssa Cristina CHELLERI

XXXV Ciclo

UNIVERSITÀ DEGLI STUDI DI GENOVA

Scuola di Scienze Mediche e Farmaceutiche

Dottorato di Ricerca in Scienze Pediatriche

Curriculum Specialità Pediatriche

Somatic *NF1* loss of heterozygosity associated with NF1-related pectus excavatum deformity: a new insight in pathogenesis?

Mutazione somatica con perdita dell'eterozigosi di deformità toracica in paziente affetto da Neurofibromatosi di tipo 1: una nuova visione della patogenesi?

Relatore:

Chiar.mo Prof. Pasquale STRIANO

Correlatore:

Dott.ssa Maria Cristina DIANA

Candidato:

Dott.ssa Cristina CHELLERI

XXXV Ciclo

Table of contents

Sinossi

La neurofibromatosi di tipo 1 (NF1) è una malattia genetica neurocutanea con un ampio spettro di segni e sintomi associati, comprese diverse anomalie scheletriche. L'associazione della NF1 con le deformità della parete toracica anteriore è stata recentemente riportata in letteratura, in particolare per quanto riguarda il pectus excavatum (PE). Nel corso degli anni, diversi autori hanno suggerito la perdita di eterozigosi (LOH) come possibile meccanismo patogenetico alla base dello sviluppo delle anomalie scheletriche tipiche della NF1.

Verrà descritto il caso di un paziente con NF1 e grave deformità toracica, in cui è stata rilevata a livello delle cellule cartilaginee la variante patogenetica germinale in eterozigosi NF1 NM_001042492.3: c.4271delC p.(Ala1424Glufs*4). Attraverso la Next Generation Sequencing (NGS), abbiamo studiato la cartilagine interessata presente all'interno del tessuto malformato del pectus excavatum e abbiamo identificato la variante frameshift aggiuntiva NM_001042492.3: c.2953delC p. (Gln985Lysfs * 7), che funge da secondo hit patogenetico come mutazione somatica del gene NF1.

L'analisi Western blot ha mostrato l'assenza di proteina NF1 wild-type nella cartilagine del paziente, coerente con un LOH somatico. Nel loro insieme, i nostri risultati supportano il ruolo di LOH nel pectus excavatum correlato alla NF1, ampliando lo spettro dei meccanismi fisiopatologici coinvolti nelle caratteristiche scheletriche correlate a NF1.

Tale meccanismo potrà essere in futuro studiato nel nostro centro e applicato ad altri tessuti anche non scheletrici (neurofibromi plessiformi, vasculopatia e molti altri). La scoperta di tale possible meccanismo patogenetico ci aiuta nella comprensione di una malattia variegata e complessa, e ci indica di prestare attenzione alla eventuale esposizione dei pazienti a fonti che emettono radiazioni ionizzanti, cercando di risparmiare esami come TC e similari.

Abstract

Neurofibromatosis type 1 (NF1) is a neurocutaneous genetic disorder with a broad spectrum of associated signs and symptoms, including skeletal anomalies. The association of NF1 with anterior chest wall deformities has been recently reported, especially the pectus excavatum (PE). Over the years, several authors have suggested loss of heterozygosity (LOH) as the possible pathogenic mechanism underlying the development of the typical NF1 skeletal features. Here, we report a NF1 patient with severe chest deformity and harboring the germline heterozygous pathogenic *NF1* variant NM_001042492.3: c.4271delC p.(Ala1424Glufs*4). Through Next Generation Sequencing (NGS), we investigated the affected cartilage from the PE deformity and identified the additional frameshift variant NM_001042492.3: c.2953delC p.(Gln985Lysfs*7), occurring as a somatic NF1 second hit mutation. Western blot analysis showed the absence of wild-type NF1 protein in the cartilage of the patient, consistent with a somatic LOH. Taken together, our findings support the role of LOH in NF1-related PE, widening the spectrum of the pathophysiological mechanisms involved in NF1-related skeletal features.

This mechanism can be studied in the future in our center and applied to other tissues, including non-skeletal tissues (plexiform neurofibromas, vasculopathy and many others). The discovery of this possible pathogenetic mechanism helps us in understanding a varied and complex disease and tells us to pay attention to the possible exposure of patients to sources emitting ionizing radiation, trying to spare exams such as CT and similar.

1. INTRODUCTION

1.1 Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen disease, is a relatively common inherited genetic disorder occurring in approximately one in 2500 to one in 3500 people worldwide, irrespective of sex or ethnic origin[1 2].

NF1 disease is a complex neurocutaneous condition with an autosomal dominant inheritance pattern, with complete penetrance and variable expressivity even within families[3(p1)].

1.1.1 Pathogenesis

The disease is caused by dominant loss-of-function mutations of the NF1 gene, located at 17q11.2. This gene encodes for a protein called Neurofibromin, which is expressed at high levels in the nervous system and it functions as a tumor suppressor, playing an important role in regulating negatively the cellular RAS-MAPK (mitogen-activated protein kinases) signaling pathway.

Neurofibromin stimulates the GTPase activity of RAS, acting as a GTPase activating protein (GAP) on RAS. Pathogenetic mutations of the NF1 gene reduces the ability of RAS to hydrolyze GTP and to shift from an active to a GDP-bound inactive state. Consequently, any *NF1* loss-of-function mutation increases cellular levels of the active GTP-bound form of RAS, leading to uncontrolled cell growth. For these reasons, patients with NF1 pathogenetic mutations have an increased risk of developing benign and malignant tumors.

Tumorigenesis probably needs a second somatic mutation to disrupt the remaining functional copy of this gene, according to the two-hit hypothesis[4]. Over 1300 different pathogenic NF1 mutations have been identified. Approximately 50% of cases of NF1 are familial and about 50% of individuals with neurofibromatosis type 1 have no family history of the disease and they develop a *de novo NF1* mutation. With the advent of accurate genetic tests, early

genotype-phenotype correlations are beginning to emerge; moreover, studies found out that people with genomic microdeletions affecting all the NF1 gene have a more severe phenotype[1].

1.1.2 Clinical features

NF1 is characterized by a highly variable inter- and intrafamilial expressivity. Although the availability of increasingly precise molecular analysis that leads to a diagnosis confirmation in about 95% of patients, the clinical diagnostic criteria, outlined in the National Institutes of Health (NIH) Consensus Development Conference in 1988, remains the gold standard for making the diagnosis. According to these criteria, two or more of the following features, reported in Table 1, are sufficient to establish a diagnosis of Neurofibromatosis type 1.

Table 1: *National Institutes of Health Neurofibromatosis type 1 Diagnostic Criteria. The diagnostic criteria require the presence of two clinical features*

USA National Institute of Health neurofibromatosis 1 diagnosti criteria
Six or more café-au-lait macules with greatest diameter of >5 mm in pre - pubertal subjects, or >15 mm in post-pubertal subjects
Two or more neurofibromas of any type, or one plexiform neurofibroma
Freckling in the axillary or inguinal region
Optic glioma
Two or more Lisch nodules (iris hamartomas)
bone cortex with or without pseudoarthrosis A first-degree relative (parent, sibling or offspring) with neurofibromatosis 1 by above criteria
A first-degree relative (parent, sibling, or child) with NF1 diagnosed using the above criteria

The diagnosis of NF1 is usually based on cutaneous symptoms, especially *café-au-lait* spots, axillary freckling, and multiple fibromas which usually appear in early childhood. More rarely the disease can involve cardiovascular and gastrointestinal systems[5(p1),6,7(p1)].

The first signs of the disease usually are cutaneous ones and Lisch nodules of the iris. More rarely NF1 can involve cardiovascular and gastrointestinal systems or cause intellectual disabilities, learning difficulties, short stature, vasculopathy, bone dysplasia, optic gliomas, and an increased risk for benign and malignant tumors[8].

The first clinical manifestations of NF1 are *café-au-lait* macules (CALMs) which usually appear at birth or in the first two years of life. These lesions have not malignant potential[9] and may be small or large, lighter, or darker and they tend to darken with sun exposure and fade with advancing age. CALMs are typically flat, uniformly hyperpigmented macules with borders well defined and they tend to increase in number and size through early childhood. The presence of more than five café-au-lait macules (>0,5 cm in diameter before puberty or >1,5 cm after puberty) is one of the diagnostic criteria for Neurofibromatosis type 1[1].

Ninety-nine percent of NF1 patients have six or more *café au lait* macules greater than 5 mm in diameter by the first years of life[9].

Axillary and inguinal freckling is a common clinical feature with no malignant potential that represents another diagnostic criterion of NF1[9].

Freckles are small pigmented macules that appear typically in the axillary and inguinal area, but they could appear in any area of the body, especially in the intertriginous areas, at the base of the neck, at the upper eyelid and under the breasts in women. More rarely, freckling may spread all over the body.

Inguinal and axillary freckling affects almost ninety percent of NF1 patients by the age of seven[3(p1)].

Neurofibromas are benign peripheral nerve sheath tumors that occur in the majority of patients as single or multiple lesions and they are one of the cardinal features of NF1. They typically increase in number with age and they are often associated with cosmetical impact or cause irritation because of rubbing or clothing irritation[10(p1)].

Neurofibromas are composed of Schwann cells, fibroblasts, endoneurial fibroblasts, masts cells, macrophages, endothelial cells, pericytes, and axons. There are five subtypes: cutaneous, subcutaneous, nodular, diffuse plexiform, and spinal[11]. Neurofibromas are present in 48% of ten years old patients and 84% of twenty years old patients[12]. Cutaneous neurofibromas are found in the vast majority (>95%) of patients with NF1. They are soft flesh-colored or purplish nodules that may become pedunculated as they grow. They usually develop during late childhood or early adolescence and do not undergo malignant transformation, but they can cause discomfort or disfigurement if they are numerous[6]. Spinal neurofibromas may cause neurologic symptoms by compressing the spinal cord or spinal roots within the foraminal spaces. They are often associated with both sensory and motor deficits[1 10(p1)].

Plexiform neurofibromas are benign tumors that arise from multiple nerve fascicles and can grow along the length of the nerve. They occur in approximately 30% of individuals with Neurofibromatosis type 1 and can cause significant morbidity in patients because, as they progress over time, they become intricately involved with the surrounding tissues[13]. They often appear early in childhood and are believed to be congenital lesions; sometimes these tumors may not be detectable on clinical examination, especially when they reside deep in the body. These tumors may be asymptomatic or can cause pain and bone destruction by compression of surrounding structures. Symptomatic lesions have to be removed surgically, but this approach is often technically impossible due to the interdigitation into normal tissues and peripheral nerves. Moreover, after surgery, they often relapse[14(p1)].

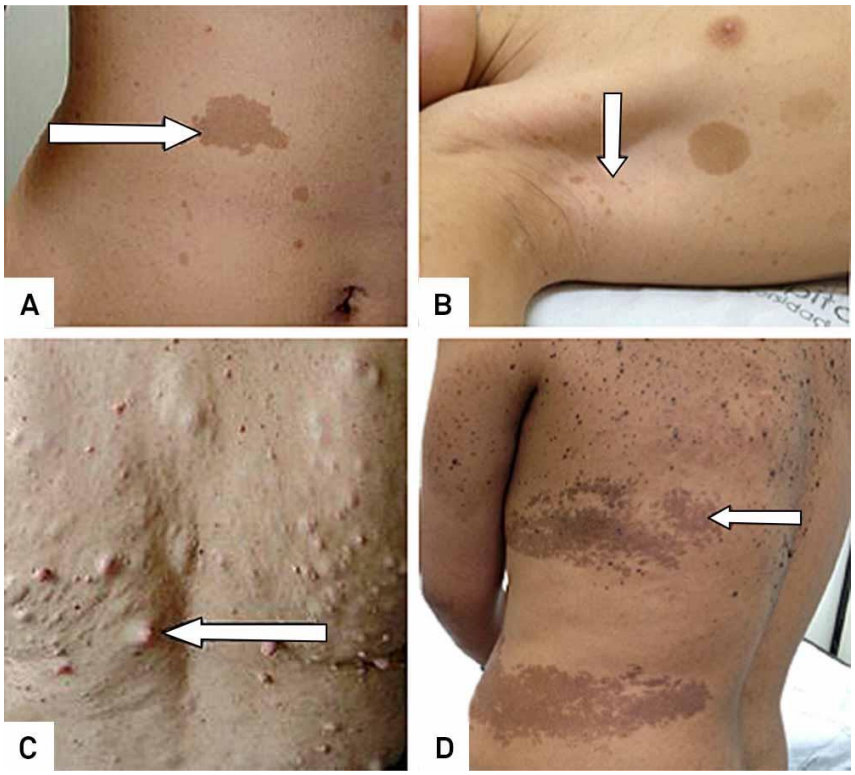


Figure 1: *Cutaneous lesions (arrows): multiple cafe au lait lesions (A); freckling and two CAL (B); Discrete neurofibromas (C); Pigmented skin lesions (epidermal nevus) misdiagnosed as CAL and freckling in a not NF1 patient*

The main difference between dermal neurofibromas and the plexiform counterparts is the risk of malignant transformation to Malignant Peripheral Nerve Sheath Tumours (MPNSTs). Pain, rapid growth, and neurologic dysfunction usually anticipate the malignant transformation[14(p1),15(p1)]. The lifetime risk of malignant transformation of plexiform neurofibromas is approximately 10%[16(p1), 17]. Malignant Peripheral Nerve Sheath Tumors (MPNST) are uncommon, biologically aggressive soft tissue sarcomas of neural origin[18,19].

Optic pathway glioma is the most common brain tumor in patients with NF1. They are usually bilateral and can involve all the optic tract; in particular the optic nerves alone (type 1), the chiasm with or without nerves involvement (type 2), hypothalamus, and other adjacent structures (type 3). Optic gliomas can both be benign and malignant lesions. In children with Neurofibromatosis type 1 these lesions typically are grade 1 pilocytic astrocytomas, benign,

indolent, and not requiring any intervention. Instead, sporadic optic pathway gliomas usually have a more aggressive natural history.

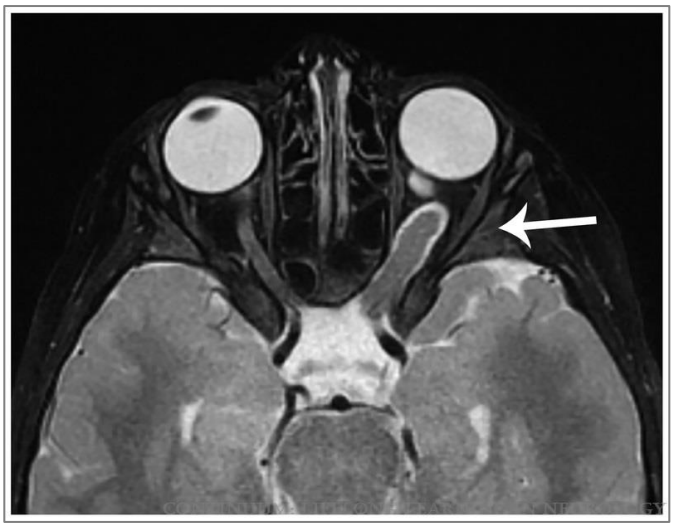


Figure 2: *axial T2-weighted fat-saturated brain MRI demonstrates a left optic pathway glioma in a 4-year-old boy with neurofibromatosis type 1*

Although many optic pathway gliomas (OPG) are asymptomatic and they are discovered accidentally with MRI, up to half of it can cause a large variety of symptoms, such as nerve compression, neural atrophy, decreased visual acuity, dyschromatopsia, nystagmus, afferent pupillary defect, visual field restriction and, when enlarging, strabismus and proptosis. Headaches and papilledema can be associated with large chiasmal and hypothalamic lesions by compression of the third ventricle and elevated intracranial pressure. More rarely OPG can cause systemic symptoms like precocious puberty or diencephalic syndrome. They affect 9.5 to 25% of patients with NF1 in the first seven years of life. The two main radiological criteria used to characterize OPG are thickening (diameter greater than 3.9 mm) and tortuosity of the nerve. The presence of contrast enhancement at the MRI is associate with more aggressive forms[20(p1),21,22,23(p1),24]

Lisch nodules are hamartomatous lesions, characteristically bilateral, well defined, dome-shaped, and gelatinous protruding above the iris surface. These lesions are usually

asymptomatic: they do not impair vision nor cause any medical problems. They can vary in number, size (2 mm of diameter typically), and color from clear to dark. They usually occur in most patients with Neurofibromatosis type1 older than ten years old. These pigmented lesions are best detected on slit-lamp examination by an experienced ophthalmologist, but they must be distinguished from iris nevi[25,26,1].

Approximately 50% of children with NF1 have important musculoskeletal manifestations; the most common findings include sphenoid wing dysplasia, dural ectasia[27], anterior chest wall deformities like pectus carinatum and excavatum, tibial/fibula pseudoarthrosis, osteopenia, and osteoporosis[28]. Scoliosis usually involves the lower cervical or the upper thoracic spine and is categorized into dysplastic and not dysplastic forms[29]. Dysplastic scoliosis can produce spinal cord compression and both acute and chronic neurologic complication, but severe dysplastic form is much less frequent than the not dysplastic one[26].

Tibial pseudoarthrosis manifests as cortical thinning and anterolateral bowing which can may lead to pathological fracture. Its presence requires an early intervention because of the high risk of fracture and development of pseudoarthrosis tissue that results from healing[30(p1)].

Tibial pseudoarthrosis usually presents within the first year of life and often it is the first recognized manifestation of NF1. Fracture is common by two years of age and pseudoarthrosis and ankle valgus develop with time[28].

Sphenoid wing dysplasia usually presents as a unilateral defect and it can cause proptosis. Most cranial defects are associated with an ipsilateral temporal-orbital plexiform neurofibroma[31].

Children with NF1 often exhibit learning disabilities, cognitive delays, and attention deficits. Neurocognitive impairment is very common in children with NF1, with a QI score often lower than 70. Learning disabilities can include visuospatial and visuomotor deficits and verbal disabilities in the realms of reading (dyslexia), spelling, vocabulary, naming, verbal reasoning,

written mathematics, and written language. Patients can also have deficits in executive functions and attention deficit (ADHD)[32(p1)].

Neurofibromatosis type 1 is associated with lots of magnetic resonance abnormalities, interesting both white and grey matter. A typical lesion of NF1 is the "undefied bright object" (UBO) that is usually discovered in the cerebellum, brainstem, basal ganglia, and thalami[33]. UBOs mainly occur in children and adolescents, tending to disappear in later age[34].

They represent non-neoplastic brain lesions with high T2-weighted signal on MRI, without mass effect, edema, or contrast enhancement[32(p1)].

The most important question concerning UBOs is their possible involvement in cognitive impairment and their impact on learning, especially in NF1 children. The literature does not provide a definitive answer, even if there is increasing evidence correlating their presence with cognitive dysfunction, especially for UBOs located in the thalamus[35].

1.1.3 Differential diagnosis

The severity and clinical features are highly variable between individuals, even within families. The variable expressivity of NF1 can make clinical diagnosis hard, especially in non-familial pediatric cases, because lots of symptoms are age-related and frequency of features increases with age. The variability of clinical expressivity in NF1 also makes it difficult to predict future manifestations of the disease in affected children[8].

For this reason genetic test is often performed: in individuals with an unclear diagnosis, to confirm suspected mosaic NF1, to establish genotype-phenotype correlation, to assist in genetic 13avourably13 and family planning. It can be helpful to do differential diagnosis – in particular among NF2, Legius syndrome, Noonan syndrome with multiple lentigines (previously called LEOPARD syndrome)[36(p1)].

The differential diagnosis is between other neurocutaneous conditions like neurofibromatosis type 2 (NF2), Legius syndrome and Noonan syndrome with multiple lentigines (previously called LEOPARD syndrome)[13].

- Neurofibromatosis type 2 is an autosomal dominant inherited disorder caused by a mutation of the *NF2* tumor suppressor gene located on chromosome 22q, with nearly 100% penetrance by 60 years of age Neurofibromatosis type 2[37].

  Patients develop nervous system tumors, peripheral neuropathy, ophthalmological lesions (cataracts, epiretinal membranes, and retinal hamartomas)[38(p2)] doi:10.1016/S0140-6736(09)60259-2, and cutaneous lesions (the main three types of skin tumors are: NF2 *plaques, nodular schwannomas* and *neurofibromas*)[39].

  Café au lait macules are found in approximately 40% of patients with NF2, but usually are smaller than NF1 ones; they tend to be paler and have more irregular margins compared to those of NF1.

  More than 95% of patients have bilateral vestibular nerve schwannomas that are the distinctive features of Neurofibromatosis type 2 and can cause tinnitus, vertigo, hearing loss, and brain stem compression. Affected people can also develop schwannomas in other cranial, spinal, and peripheral nerves and ophthalmological lesions like cataracts and epiretinal membranes. Other nervous system tumors associated with the disorder include meningiomas and glial cells tumors like ependymomas and astrocytomas[40];

- Schwannomatosis is a dominantly inherited disorder caused by germline mutations of either *SMARCB1* or *LZTR1* tumor suppressor genes[41,42]. The disease is characterized by multiple schwannomas, most commonly affecting the spine (74%) and the peripheral nerves (89%), while cranial nerves schwannomas (mostly trigeminal) are uncommon. Skin manifestations

are limited to schwannomas and there are not ocular features. Most patients present with pain, which is the most common feature reported[43,44].

- Legius syndrome is an autosomal dominant inherited disorder caused by a mutation in the SPRED1 gene, with a complete penetrance[45].

  Approximately 5% of patients meet the National Institutes of Health diagnostic criteria for neurofibromatosis type 1[13].

  This condition causes *café au lait* macules with or without axillary and inguinal freckling. Learning disabilities and ADHD are often present but they usually are less severe than NF1. Lisch nodules, optic pathway gliomas, neurofibromas, tibial dysplasia and malignant peripheral nerves are usually absent[45,46].

  The distinction between NF1 and Legius syndrome in young children whit out family history can only be performed with genetic tests[47].

- Noonan syndrome with multiple lentigines is a rare multisystem RASopathy caused by mutations of PTPN11, RAF1, and BRAF. The disease was previously called LEOPARD syndrome, an acronym for its cardinal features of multiples Lentigines, Electrocardiographic conduction abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retardation of growth, and sensorineural Deafness[48,49].

  When employed, NF1 mutation analysis is 95% sensitive[7(p1)].

1.1.4 Prognosis

Despite the high prevalence of NF1, there is not much information about mortality. Studies have shown a shorter lifetime in people affected by NF1 compared to the general population,

especially in probands. The mean age at death of people with NF1 is probably 15 years lower than the mean age at death in the rest of the population.

The most common causes of morbidity and mortality are malignant neoplasms, vascular disease, cerebrovascular disease, scoliosis, epilepsy, and mental retardation. Malignant neoplasms, in particular, are the principal cause of death in people with NF1[50].

Moreover, patients with NF1 have a higher risk of connective tissue tumors, central nervous system tumors, gastrointestinal stromal tumors, breast cancer, leukemia, and neuroendocrinal tumors as pheochromocytoma[51].

Table 2: *lifetime risk of cancer in children with NF1*[1]

Tumor	Lifetime risk
Glioma of the Optic Pathway	15-20%
Other brain tumors	More than fivefold increase
Malignant peripheal nerve-sheat tumor	8-13%
Gastrointestinal tumor	4-25%
Breast cancer	About fivefold increase
Leukaemia	About sevenfold increase
Phaeochromocytoma	0.1-5.7%
Duodenal carcinoid tumor	1%
Rhabdomyosarcoma	1.4-6%

2. NF1 AND SKELETAL ANOMALIES

Neurofibromatosis type 1 (NF1) is a complex neurocutaneous condition caused by pathogenic variants in the NF1 gene (OMIM * 613113) and characterized by a heterogeneous clinical presentation[52]. So far, genotype-phenotype correlations remain elusive in NF1 and this is especially true for skeletal abnormalities, although these lesions are common in NF1 patients[53]. NF1-related skeletal defects include a broad range of manifestations, such as osteoporosis, short stature, dysplasia of the tibia and other long bones, vertebral defects, sphenoid wings dysplasia, and anterior chest wall deformities[54]. Of note, the latter have been only recently reported in association with NF1, especially *pectus excavatum* (PE)[54]. This consists of a depression in the anterior chest wall resulting from a dorsal deviation of the sternal bone and 3rd-7th rib or costal cartilage[55-57]. PE is the most common chest wall deformity (90% of all cases) and has been recently found to be especially frequent in NF1 patients, with a higher incidence as compared to the general population[58-60].

The pathogenic mechanism underlying NF1-related skeletal abnormalities remains elusive. Previous studies have suggested that the somatic loss of heterozygosity (LOH) in the *NF1* gene may contribute to the development of these defects[61-64]. This mechanism is relevant for cancerogenesis and LOH of essential genes accounts for potential cancer vulnerabilities[65]. LOH of *NF1* has been identified in cells extracted from skeletal and nervous tissues in affected individuals, such as tibial pseudoarthrosis, dystrophic scoliosis, or plexiform neurofibroma samples[61-64]. Furthermore, mouse models have been found to recapitulate in part the bone abnormalities observed in NF1 patients, helping clarify that *NF1* haploinsufficiency accounts for the generalized bone remodeling defects, while the complete loss due to LOH is responsible for the focal defects, such as the dysplasia[54,64].

In this study, we thoroughly investigated a cartilage sample from a PE deformity in a subject with NF1 caused by a germinal NF1 frameshift variant. The combination of different techniques allowed to identify a somatic LOH in the affected cartilage leading to the complete absence of the wild-type NF1 protein.

3 MATERIALS AND METHODS

3.1 Ethical approval and clinical examination

The study was conducted in accordance with the Declaration of Helsinki and ethical approval was obtained by the 'Comitato Unico Regionale Regione Liguria', Genoa, Italy. Informed consent was waived for this study as all clinical and radiological information has been anonymized. The patient was thoroughly evaluated through specialistic pediatric and surgical assessment. Imaging studies were reviewed by experts in thoracic disorders of childhood.

3.2 Biopsy and pathology examination

A sample of affected cartilage was obtained from the central core of the tissue corresponding to the PE deformity. After surgery, a cylindrical fragment of discarded cartilage measuring 1 x 1 cm was frozen to allow genetic and histo-enzymatic investigations. It was then thawed, fixed in 10% buffered formalin, and embedded in paraffin in two blocks. Histochemical stains were performed using PAS (AB pH2.5; AB pH1).

3.3 Next Generation Sequencing

DNA was extracted from peripheral blood samples and from discarded surgical frozen tissue using commercial kits. A next generation sequencing (NGS) custom-designed panel was created using the Ion AmpliSeq™ Designer v6.13 algorithm provided by Thermo Fisher Scientific (Carlsbad, CA, USA) in order to target the entire coding sequence (CDS) and 10 bases of the adjacent intronic regions of NF1 gene (NM_001042492.3). NGS sequencing was performed on genomic DNA extracted from both blood and cartilaginous biopsy using the Ion Gene Studio S5 platform (Thermo Fisher Scientific, Inc.). Variants were analyzed using both

the Ion Reporter Software v.5.6 (Thermo Fisher Scientific, Inc.) and the CLC Genomics Workbanch 6.5.1 software (Qiagen).

3.4 Multiplex Ligation-Dependent Probe Amplification

Multiplex Ligation-Dependent Probe Amplification (MLPA) was performed in order to detect somatic exonic deletions of NF1 using two commercial kits, the SALSA P081/P082 kits (MRC-Holland, Amsterdam, The Netherlands). Both cartilage and peripheral blood were also tested by high-resolution oligonucleotide array CGH (Comparative Genomic Hybridization) using the 4x180 K Kit, with probe design 086332 (Agilent 119 Technologies, Santa Clara, CA), according to the manufacturer's instructions, in order to detect large somatic LOH inclusive of NF1.

3.5 Western blot

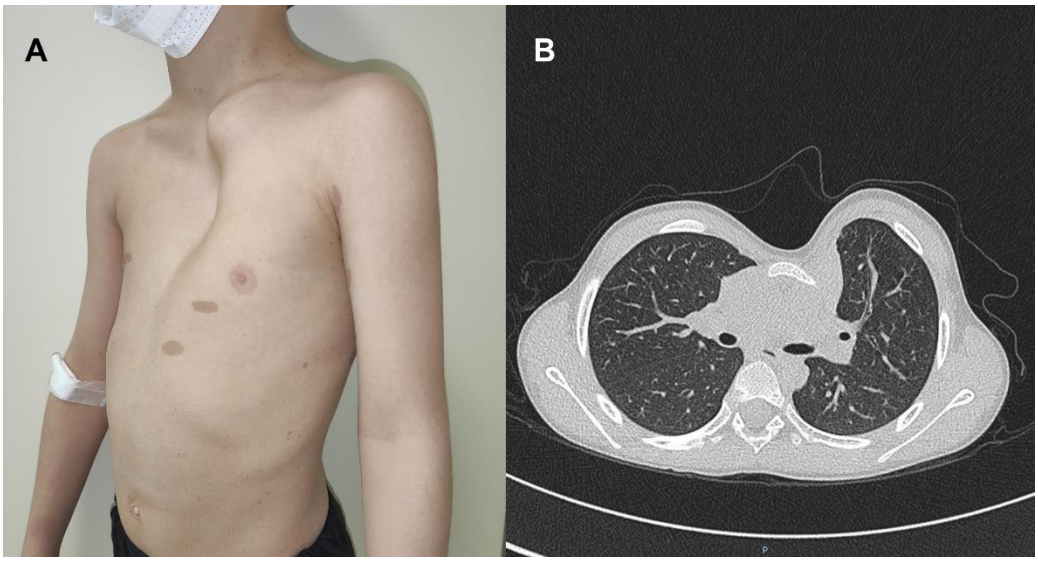
NF1 frameshift mutations that generate premature termination codons (PTCs) lead to the synthesis of truncated neurofibromin. To study the effects of NF1 causative mutations at the protein level on the pathological tissue, we performed a Western Blot (WB) analysis. Fragments of the abnormal and autoptic control cartilage samples were pulverized in liquid nitrogen, lysed and analyzed by WB to evaluate NF1 protein expression (Supplementary Material). Using a primary Ab against the N-terminus domains of neurofibromin, a specific band of about 260 kDa was detected in the total lysates from control cartilage samples.

4. RESULTS

4.1 Clinical study

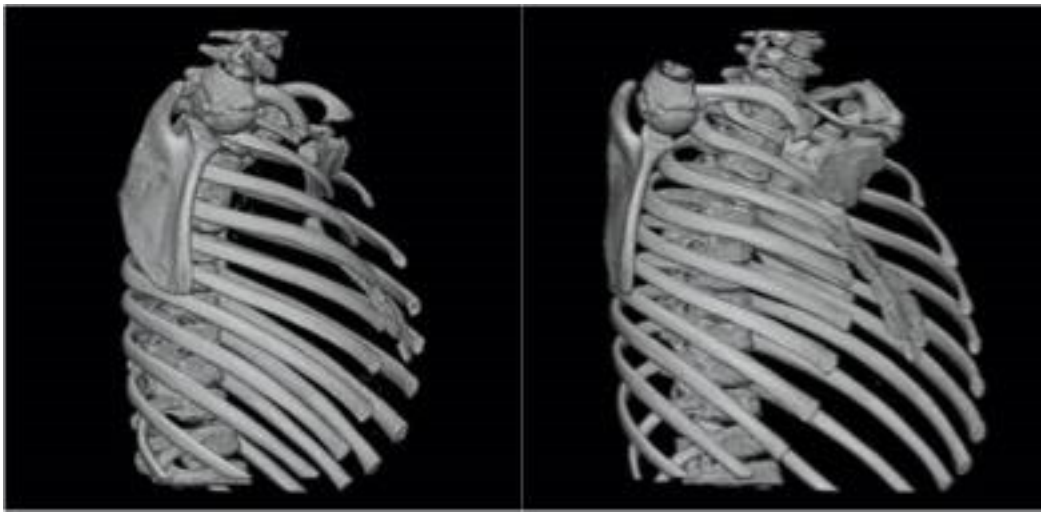
The patient was diagnosed with NF1 at the age of 2 years, when he presented with cafè-au-lait macules and Lisch nodules. Over the years, he developed a plexiform neurofibroma in the left popliteal fossa and brain-MRI at the age of 5 years showed unidentified bright objects (UBOs), thus confirming a clinical NF1 diagnosis. At the age of 4 years, the patient was referred to our Institution for a rapidly enlarging deformity of the anterior chest wall. Clinical examination revealed a severe PE deformity, consisting of a severe depression of the sternal manubrium associated with marked thoracic asymmetry and marked protrusion of the left costal cartilages (Fig.3).

Fig. 3 Clinical and imaging findings. *A) Clinical photograph showing the deformity of the anterior chest wall and some typical cafè-au-lait macules. The PE deformity consists of a severe dorsal deviation of the sternal manubrium and costal cartilage. B) Axial CT-scan of the chest showing the sternal depression and the deformation of the costal cartilage.ù*



Thoracic CT-scan confirmed the severe bony depression between the manubrium and the body of the sternum, which led to the compression of the right ventricle and supra-aortic vessels (Fig.4).

Figure 4. *3D computed tomography reconstruction of the anterior chest wall showing the severe dorsal deviation of the manubrium and the sternal bone.*



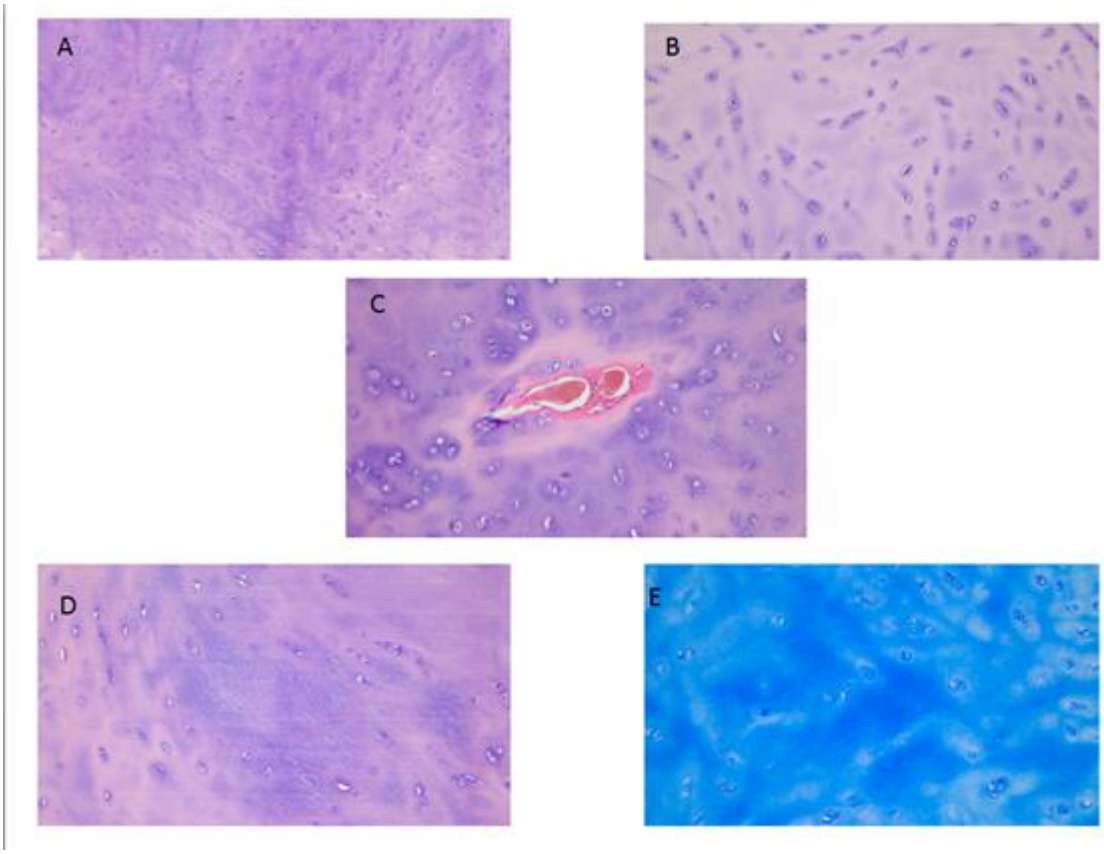
The patient underwent a major elective surgery combining minimal Invasive Repair of PE/MIRPE (Nuss procedure) and open reconstruction of thoracic wall, with good overall outcome.

4.2 Pathology

The histological examination revealed a mature type cartilage (Ki67-negative) with isogenic groups distributed in an abundant amorphous matrix. The endochondral vascularization network was normal. A nucleus of accentuated condrification with initial degeneration of the matrix around the largest chondrocytes with prominent nucleus was seen. The intercellular

matrix, where more abundant, presented accentuated basophilia suggestive of mild degenerative phenomena (Fig.5).

Fig. 5 Pathology analysis. *The cartilage extracted from the PE deformity is of mature type. B) The chondrocytes appear lightly more immature at the periphery. C) The endochondral vascularization network is normal. D-E) The isogenic groups are distributed in an abundant amorphous matrix (AB pH2.5)*

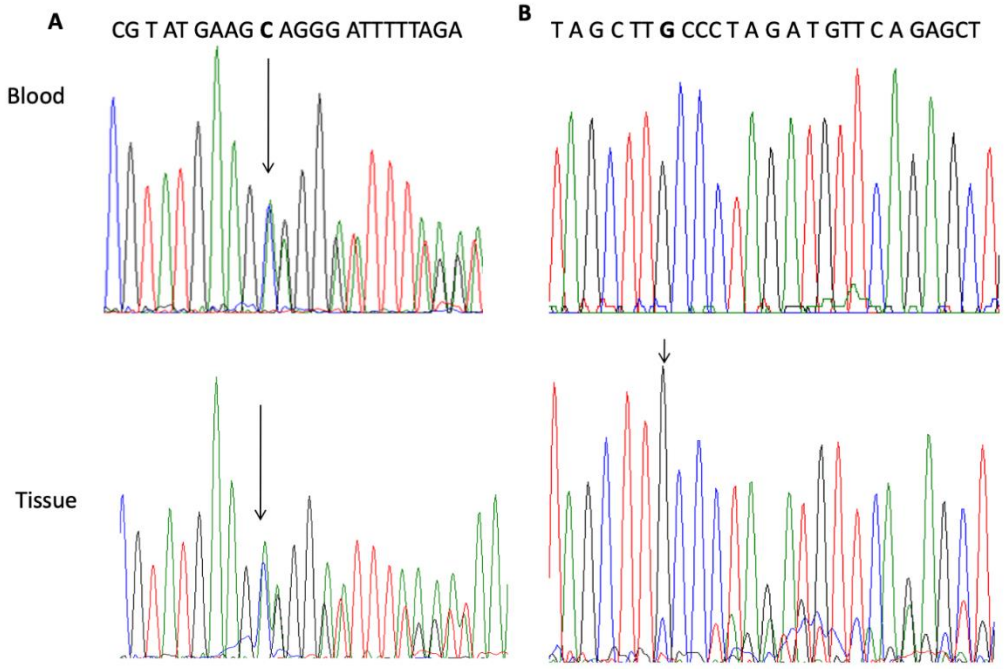


Histochemical stains (PAS; AB pH2.5; AB pH1) confirmed the presence of outbreaks of the mentioned degeneration with excessive accumulations of acidic mucopolysaccharides (chondroitin sulphate)[66]. The chondrocytes appeared slightly immature at the periphery but lacked fetal cartilage features, which was also confirmed by immunohistochemical stains.

4.3 Genetic investigation

We identified a germline pathogenic variant c.4271delC p.(Ala1424Glufs*4) present both in blood (variant allele frequency 48%, coverage 341X) and in the pathological tissue (variant allele frequency 38%; coverage 531X). This variant is absent in gnomAD and considered pathogenic according to the ACMG/AMP guidelines, as it is predicted to lead to a truncated transcript or nonsense-mediated mRNA decay (NMD). In the tissue, we identified a somatic second hit in NF1, the c.2953delC p.(Gln985Lysfs*7), with a variant allelic frequency of 18%. This variant, a frameshift resulting in a stop gain 7 codons after the mutation, is not present in public databases and it is predicted to be "likely pathogenic". Both the germline and the somatic variants were validated by Sanger sequencing (Figure 6).

Fig. 6 Electropherogram traces of variants confirmed by Sanger. *Sanger traces of A) germline heterozygous NF1 c.4271delC variant present both in blood and in the tissue and B) somatic NF1 c.2953delC (allelic frequency 18%) present only in the pathological tissue sample (reverse sequence) (B).*

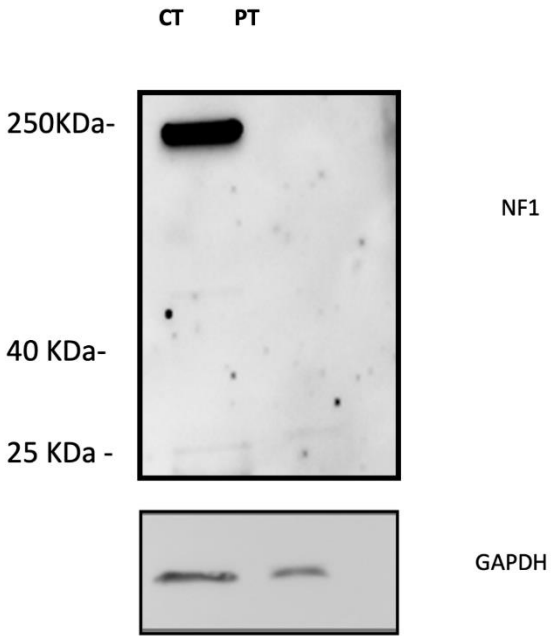


The MLPA assays excluded somatic loss of the *NF1* wild-type allele by copy number variations (data not shown). These data therefore support the assumption that the pectus excavatum of this patient was a manifestation of NF1 and was driven by a copy neutral LOH in the pathological tissue.

4.4 Western blot

No signal was instead detected in the patient's cartilage, demonstrating a complete loss of wild-type neurofibromin (Figure 7).

Fig. 7 Western blot analysis of Neurofibromin. *WB images showing immunodetection of NF1 (top) and GAPDH (bottom, as a loading control) in total lysates from control (CT) and patient cartilage samples (PT). Control cartilage sample was obtained from autoptic biopsy of a sex- and age-matched control. In the patient's cartilage, no signal was detected, consistent with a complete loss of NF1 protein expression.*



The theoretical molecular weight estimated for the two truncated form of neurofibromin resulting from the mutated alleles of our patient were 108 and 164 KDa. However, no truncated protein was detected in the assay. It is likely that mutations determining mRNAs with PTCs can render the mutant transcripts susceptible to degradation by the nonsense-mediated mRNA decay machinery, rendering the protein undetectable.

5. DISCUSSION

Skeletal manifestations are particularly common and heterogeneous in NF1 patients[54]. Of note, in comparison to the general population, these subjects show a higher incidence of anterior chest wall deformities, especially PE[58], even in the pediatric age range[59]. However, the pathogenic mechanisms underlying these developmental abnormalities remained elusive. LOH of *NF1* has been implicated in other NF1 manifestations. In 2009, Steinmann et al. published the results of a LOH analysis study conducted on 43 plexiform neurofibromas from 31 NF1 patients[63]. The authors observed LOH involving 17q markers in a total of 13 (30%) tumors[63]. Some evidence in favor of the role of a somatic LOH in the pathogenesis of NF1-related skeletal manifestations has been provided by a few previous studies, which focused on the possible role of the double inactivation of the *NF1* gene in the affected tissues[54].

In 2006, Stevenson et al. reported two cases of tibial pseudoarthrosis associated with a double *NF1* inactivation[61]. This was caused by the combination of germinal stop gain variants (NM_000267.3: c.2446C>T (p.Arg816*) and c.7846C>T (p.Arg2616*)) and somatic LOH of *NF1* in the abnormal tissue of affected individuals, as suggested by allele imbalance[61]. The authors concluded that the loss of *NF1* function causes a dysregulation of the *Ras* pathway, which leads to an impairment of the differentiation and proliferation of osteoblasts and osteoprogenitors[61]. In a further study, the somatic LOH for most of the 17q region was detected in spinal samples from two NF1 patients with dystrophic scoliosis, who harbored germinal splicing (NM_000267.3: c.6642-1G>T or frameshift (NM_000267.3: c.4953_4965del (p.Asp1651Glufs*22)) variants[62].

The role of LOH in relation to NF1-related skeletal manifestations was also investigated through animal models. Wang et al. focused on the study of the role of the loss of *NF1* in osteo-chondro-progenitors in the development of *NF1*-related skeletal manifestations[64]. Crossing the *Col2a1*(collagen, type II, alpha 1)-*Cre* promoter mouse with

the *Nf1*$^{flox/flox}$ mouse, the authors found that the *Nf1/Col2*$^{-/-}$ mice showed progressive scoliosis, tibial pseudoarthrosis, and skeletal abnormalities involving the skull and anterior chest wall, demonstrating that loss of *Nf1*in axial and appendicular osteo-chondro-progenitors recapitulates the skeletal abnormalities of NF1 patients[64]. Taken together, these findings suggest that the somatic LOH of the *NF1* gene may be involved in the pathogenesis of NF1-related skeletal abnormalities, although this should be still considered multifactorial[54].

In our patient, we detected the *de novo* germline frameshift variant p.(Ala1424Glufs*4) in *NF1* and a second, somatic and *de novo* frameshift variant p.(Gln985Lysfs*7). Although it was not possible to assess if this second variant occurred in *cis* or in *trans* to the germline variant, the western blot revealed absence of wild-type neurofibromin in the abnormal tissue. This stands in favor of the occurrence of the second variant in the wild type allele of *NF1*, thus in trans with the germline variant. This finding confirms a somatic LOH in the affected cartilage. Accordingly, in line with previous studies about other NF1-related skeletal features, our observation further supports the role of LOH in the pathogenesis of PE in NF1 patients. Whether the Ras/Erk constitutive activation caused by the complete loss of NF1 function or additional molecular mechanisms are implicated in the development of these skeletal alterations remains to be elucidated[54,64]. Further studies will play a crucial role in the clarification of this aspect. However, our observations confirm that somatic double hits may be critically involved in the development of diverse clinical manifestations NF1, leading to suggest that the adoption of radiation-sparing approaches might be advisable in the diagnosis and management of affected individuals.

Our study had some limitations related to the availability and processing of the abnormal tissue analyzed. First, the cartilage samples were obtained from discarded tissue from surgical procedures, which was initially stored for future studies. Thus, the identification of the affected tissue relied on the judgment of surgeons and pathologists and was based on

the labels indicated on specimen containers. Second, the DNA employed for genetic studies was extracted from frozen heterogeneous samples containing a mixed cellular population. Then, we specifically obtained pathological tissue from the central core of the abnormal cartilage of the pectus excavatum and, therefore, we were not able to investigate cells from the peripheral regions of the malformation. Since the second variant occurred as a somatic change, the abnormal tissue is predicted to be a mosaic and it is possible that other cells within the PE have traces of wild-type NF1. Eventually, we could not perform exome sequencing on the sample and thus, for example, we cannot exclude the presence of possible variants in modifier genes. Indeed, it is possible that other genetic factors may influence the pathogenic role of the somatic LOH that we observed in relation to the abnormal morphogenesis of the analyzed cartilage.

6 CONCLUSIONS

In this study, we detected a somatic double inactivation of the *NF1* gene in the cartilage tissue from a PE deformity in a subject with NF1 due to a germline frameshift variant. Tissue analysis revealed that this neutral LOH was due to a somatic double hit frameshift variant resulting in a premature termination codon. Accordingly, the biallelic inactivation of *NF1* resulted in no wild-type neurofibromin in the affected tissue. In line with the limited previous studies, our findings suggest that LOH is a relevant contributing factor in the pathogenesis of skeletal abnormalities in NF1, including anterior chest wall deformities. Considering the possible overlapping pathophysiological mechanisms, this would lead to speculate about the possible use of MEK inhibitors in NF1 patients with severe skeletal abnormalities, which demonstrated effective in patients with NF1-associated tumors and plexiform neurofibromas. Further studies will be crucial to delineate the underlying pathogenic mechanisms and shed light on the possible involvement of similar alterations in other NF1-related extra-neurological manifestations.

This mechanism can also be studied in the future in our center and applied to other tissues, including non-skeletal tissues (plexiform neurofibromas, vasculopathy and many others).

The discovery of this possible pathogenetic mechanism helps us in understanding a varied and complex disease and tells us to pay attention to the possible exposure of patients to sources emitting ionizing radiation, trying to spare exams such as CT and similar.

References

1. Hirbe AC, Gutmann DH. Neurofibromatosis type 1: a multidisciplinary approach to care. *The Lancet Neurology*. 2014;13(8):834-843. doi:10.1016/S1474-4422(14)70063-8

2. Anderson JL, Gutmann DH. Neurofibromatosis type 1. In: *Handbook of Clinical Neurology*. Vol 132. Elsevier; 2015:75-86. doi:10.1016/B978-0-444-62702-5.00004-4

3. Boyd KP, Korf BR, Theos A. Neurofibromatosis type 1. *Journal of the American Academy of Dermatology*. 2009;61(1):1-14. doi:10.1016/j.jaad.2008.12.051

4. NISC Comparative Sequencing Program, NCI DCEG Cancer Genomics Research Laboratory, Pemov A, et al. The primacy of NF1 loss as the driver of tumorigenesis in neurofibromatosis type 1-associated plexiform neurofibromas. *Oncogene*. 2017;36(22):3168-3177. doi:10.1038/onc.2016.464

5. Rasmussen SA, Friedman JM. NF1 Gene and Neurofibromatosis 1. *American Journal of Epidemiology*. 2000;151(1):33-40. doi:10.1093/oxfordjournals.aje.a010118

6. NF France Network, Bergqvist C, Servy A, et al. Neurofibromatosis 1 French national guidelines based on an extensive literature review since 1966. *Orphanet J Rare Dis*. 2020;15(1):37. doi:10.1186/s13023-020-1310-3

7. Anderson JL, Gutmann DH. Neurofibromatosis type 1. In: *Handbook of Clinical Neurology*. Vol 132. Elsevier; 2015:75-86. doi:10.1016/B978-0-444-62702-5.00004-4

8. Kang E, Kim Y-M, Seo GH, et al. Phenotype categorization of neurofibromatosis type I and correlation to NF1 mutation types. *J Hum Genet*. 2020;65(2):79-89. doi:10.1038/s10038-019-0695-0

9. DeBella K, Szudek J, Friedman JM. Doi:10.1542/peds.105.3.608. *Pediatrics*. 2000;105(3):608-614. doi:10.1542/peds.105.3.608

10. Isenberg JC, Templer A, Gao F, Titus JB, Gutmann DH. Attention Skills in Children With Neurofibromatosis Type 1. *J Child Neurol*. 2013;28(1):45-49. doi:10.1177/0883073812439435

11. Rozza-de-Menezes RE, Brum C de AI, Gaglionone NC, et al. Prevalence and clinicopathological characteristics of lipomatous neurofibromas in neurofibromatosis 1: An investigation of 229 cutaneous neurofibromas and a systematic review of the literature. *J Cutan Pathol*. 2018;45(10):743-753. doi:10.1111/cup.13315

12. DeBella K, Szudek J, Friedman JM. Use of the National Institutes of Health Criteria for Diagnosis of Neurofibromatosis 1 in Children. *Pediatrics*. 2000;105(3):608-614. doi:10.1542/peds.105.3.608

13. Rosser T. Neurocutaneous Disorders: *CONTINUUM: Lifelong Learning in Neurology*. 2018;24(1):96-129. doi:10.1212/CON.0000000000000562

14. Miller DT, Freedenberg D, Schorry E, et al. Health Supervision for Children With Neurofibromatosis Type 1. *Pediatrics*. 2019;143(5):e20190660. doi:10.1542/peds.2019-0660

15. Cimino PJ, Gutmann DH. Neurofibromatosis type 1. *Handb Clin Neurol*. 2018;148:799-811. doi:10.1016/B978-0-444-64076-5.00051-X

16. Uusitalo E, Rantanen M, Kallionpää RA, et al. Distinctive Cancer Associations in Patients With Neurofibromatosis Type 1. *JCO*. 2016;34(17):1978-1986. doi:10.1200/JCO.2015.65.3576

17. Hagizawa H, Nagata S, Wakamatsu T, et al. Malignant peripheral nerve-sheath tumors in an adolescent patient with mosaic localized NF1: A case report. *mol clin onc*. Published online December 17, 2019. doi:10.3892/mco.2019.1969

18. Kim A, Stewart DR, Reilly KM, Viskochil D, Miettinen MM, Widemann BC. Malignant Peripheral Nerve Sheath Tumors State of the Science: Leveraging Clinical and

Biological Insights into Effective Therapies. *Sarcoma*. 2017;2017:1-10. doi:10.1155/2017/7429697

19. Farid M, Demicco EG, Garcia R, et al. Malignant Peripheral Nerve Sheath Tumors. *The Oncologist*. 2014;19(2):193-201. doi:10.1634/theoncologist.2013-0328

20. Mehlan J, Schüttauf F, Salamon JM, Kordes U, Friedrich RE, Mautner V-F. Manifestations and Treatment of Adult-onset Symptomatic Optic Pathway Glioma in Neurofibromatosis Type 1. *Anticancer Res*. 2019;39(2):827-831. doi:10.21873/anticanres.13181

21. Rasool N, Odel JG, Kazim M. Optic pathway glioma of childhood: *Current Opinion in Ophthalmology*. 2017;28(3):289-295. doi:10.1097/ICU.0000000000000370

22. Eid H, Crevier-Sorbo G, Aldraihem A, Menegotto F, Wilson N. Neurofibromatosis Type 1: Description of a Novel Diagnostic Scoring System in Pediatric Optic Nerve Glioma. *American Journal of Roentgenology*. 2019;212(4):892-898. doi:10.2214/AJR.18.20044

23. Cimino PJ, Gutmann DH. Neurofibromatosis type 1. In: *Handbook of Clinical Neurology*. Vol 148. Elsevier; 2018:799-811. doi:10.1016/B978-0-444-64076-5.00051-X

24. Fisher MJ, Avery RA, Allen JC, et al. Functional outcome measures for NF1-associated optic pathway glioma clinical trials. *Neurology*. 2013;81(Issue 21, Supplement 1):S15-S24. doi:10.1212/01.wnl.0000435745.95155.b8

25. Lewis RA, Riccardi VM. von Recklinghausen Neurofibromatosis. *Ophthalmology*. 1981;88(4):348-354. doi:10.1016/S0161-6420(81)35034-9

26. Friedman JM. Review Article : Neurofibromatosis 1: Clinical Manifestations and Diagnostic Criteria. *J Child Neurol*. 2002;17(8):548-554. doi:10.1177/088307380201700802

27. Derdabi I, El Jouadi H, Edderai M. Dural ectasia: a manifestation of type 1 neurofibromatosis. *Pan Afr Med J*. 2018;31. doi:10.11604/pamj.2018.31.226.9797

28. Vitale MG, Guha A, Skaggs DL. Orthopaedic Manifestations of Neurofibromatosis in Children: An Update: *Clinical Orthopaedics and Related Research*. 2002;401:107-118. doi:10.1097/00003086-200208000-00013

29. Margraf RL, VanSant-Webb C, Mao R, et al. NF1 Somatic Mutation in Dystrophic Scoliosis. *J Mol Neurosci*. 2019;68(1):11-18. doi:10.1007/s12031-019-01277-0

30. Stevenson DA, Birch PH, Friedman JM, et al. Descriptive analysis of tibial pseudarthrosis in patients with neurofibromatosis 1. *American Journal of Medical Genetics*. 1999;84(5):413-419. doi:10.1002/(SICI)1096-8628(19990611)84:5<413::AID-AJMG5>3.0.CO;2-1

31. Elefteriou F, Kolanczyk M, Schindeler A, et al. Skeletal abnormalities in neurofibromatosis type 1: Approaches to therapeutic options. *Am J Med Genet*. 2009;149A(10):2327-2338. doi:10.1002/ajmg.a.33045

32. Rosser TL, Packer RJ. Neurocognitive dysfunction in children with neurofibromatosis type 1. *Curr Neurol Neurosci Rep*. 2003;3(2):129-136. doi:10.1007/s11910-003-0064-3

33. Billiet T, Mädler B, D'Arco F, et al. Characterizing the microstructural basis of "unidentified bright objects" in neurofibromatosis type 1: A combined in vivo multicomponent T2 relaxation and multi-shell diffusion MRI analysis. *NeuroImage: Clinical*. 2014;4:649-658. doi:10.1016/j.nicl.2014.04.005

34. Pondé Rodrigues AC, Ferraz-Filho JRL, Torres US, et al. Is Magnetic Resonance Spectroscopy Capable of Detecting Metabolic Abnormalities in Neurofibromatosis Type 1 That Are Not Revealed in Brain Parenchyma of Normal Appearance? *Pediatric Neurology*. 2015;52(3):314-319. doi:10.1016/j.pediatrneurol.2014.11.014

35. Baudou E, Nemmi F, Biotteau M, Maziero S, Peran P, Chaix Y. Can the Cognitive Phenotype in Neurofibromatosis Type 1 (NF1) Be Explained by Neuroimaging? A Review. *Front Neurol*. 2020;10:1373. doi:10.3389/fneur.2019.01373

36. Ly KI, Blakeley JO. The Diagnosis and Management of Neurofibromatosis Type 1. *Medical Clinics of North America*. 2019;103(6):1035-1054. doi:10.1016/j.mcna.2019.07.004

37. Evans DGR. In: *Handbook of Clinical Neurology*. Vol 132. Elsevier; 2015:87-96. doi:10.1016/B978-0-444-62702-5.00005-6

38. Asthagiri AR, Parry DM, Butman JA, et al. Neurofibromatosis type 2. *The Lancet*. 2009;373(9679):1974-1986. doi:10.1016/S0140-6736(09)60259-2

39. Ardern-Holmes S, Fisher G, North K. Neurofibromatosis Type 2: Presentation, Major Complications, and Management, With a Focus on the Pediatric Age Group. *J Child Neurol*. 2017;32(1):9-22. doi:10.1177/0883073816666736

40. Asthagiri AR, Parry DM, Butman JA, et al. Neurofibromatosis type 2. *The Lancet*. 2009;373(9679):1974-1986. doi:10.1016/S0140-6736(09)60259-2

41. Kehrer-Sawatzki H, Farschtschi S, Mautner V-F, Cooper DN. The molecular pathogenesis of schwannomatosis, a paradigm for the co-involvement of multiple tumour suppressor genes in tumorigenesis. *Hum Genet*. 2017;136(2):129-148. doi:10.1007/s00439-016-1753-8

42. Evans DG, Bowers NL, Tobi S, et al. Schwannomatosis: a genetic and epidemiological study. *J Neurol Neurosurg Psychiatry*. 2018;89(11):1215-1219. doi:10.1136/jnnp-2018-318538

43. Plotkin S, Wick A. Neurofibromatosis and Schwannomatosis. *Semin Neurol*. 2018;38(01):073-085. doi:10.1055/s-0038-1627471

44. Kresak J, Walsh M. Neurofibromatosis: A Review of NF1, NF2, and Schwannomatosis. *J Pediatr Genet*. 2016;05(02):098-104. doi:10.1055/s-0036-1579766

45. Giugliano T, Santoro C, Torella A, et al. Clinical and Genetic Findings in Children with Neurofibromatosis Type 1, Legius Syndrome, and Other Related Neurocutaneous Disorders. *Genes*. 2019;10(8):580. doi:10.3390/genes10080580

46. Denayer E, Chmara M, Brems H, et al. Legius syndrome in fourteen families. *Hum Mutat*. 2011;32(1):E1985-E1998. doi:10.1002/humu.21404

47. Rodrigues LOC, Batista PB, Goloni-Bertollo EM, et al. Neurofibromatoses: part 1 ? diagnosis and differential diagnosis. *Arq Neuro-Psiquiatr*. 2014;72(3):241-250. doi:10.1590/0004-282X20130241

48. Aoki Y, Niihori T, Inoue S, Matsubara Y. Recent advances in RASopathies. *J Hum Genet*. 2016;61(1):33-39. doi:10.1038/jhg.2015.114

49. Santoro C, Pacileo G, Limongelli G, et al. LEOPARD syndrome: clinical dilemmas in differential diagnosis of RASopathies. *BMC Med Genet*. 2014;15(1):44. doi:10.1186/1471-2350-15-44

50. Mortality in neurofibromatosis 1: an analysis using U.S. death certificates. - Abstract - Europe PMC. Accessed May 28, 2020. https://europepmc.org/article/pmc/pmc1226092

51. Walker L, Thompson D, Easton D, et al. A prospective study of neurofibromatosis type 1 cancer incidence in the UK. *Br J Cancer*. 2006;95(2):233-238. doi:10.1038/sj.bjc.6603227

52. Jett K, Friedman JM. Clinical and genetic aspects of neurofibromatosis 1. *Genet Med*. 2010;12(1):1-11.

53. Scala M, Schiavetti I, Madia F, Chelleri C, Piccolo G, Accogli A, Riva A, Salpietro V, Bocciardi R, Morcaldi G, Di Duca M, Caroli F, Verrico A, Milanaccio C, Viglizzo G, Traverso M, Baldassari S, Scudieri P, Iacomino M, Piatelli G, Minetti C, Striano P, Garrè ML, De Marco P, Diana MC, Capra V, Pavanello M, Zara F. Genotype-Phenotype Correlations in Neurofibromatosis Type 1: A Single-Center Cohort Study. *Cancers*. 2021;13(8):1879.

54. Elefteriou F, Kolanczyk M, Schindeler A, Viskochil DH, Hock JM, Schorry EK, Crawford AH, Friedman JM, Little D, Peltonen J, Carey JC, Feldman D, Yu X, Armstrong L, Birch P, Kendler DL, Mundlos S, Yang FC, Agiostratidou G, Hunter-Schaedle K, Stevenson DA. Skeletal abnormalities in neurofibromatosis type 1: approaches to therapeutic options. *Am J Med Genet A*. 2009;149A(10):2327-38.

55. Brochhausen C, Turial S, Müller FK, Schmitt VH, Coerdt W, Wihlm JM, Schier F, Kirkpatrick CJ. Pectus excavatum: history, hypotheses and treatment options. *Interact Cardiovasc Thorac Surg*. 2012;14(6):801-6.

56. Robert E. Kelly, Robert C. Shamberger. Congenital Chest Wall Deformities, Pediatric Surgery 7th ed. 2012, pp. 779-808.

57. Brochhausen C, Turial S, Müller FK, Schmitt VH, Coerdt W, Wihlm JM, Schier F, Kirkpatrick CJ. Pectus excavatum: history, hypotheses and treatment options. Interact Cardiovasc Thorac Surg. 2012 Jun;14(6):801-6.

58. Miraglia E, Chello C, Calvieri S, Giustini S. Pectus excavatum in neurofibromatosis type 1: single-center experience and review of the literature. *G Ital Dermatol Venereol*. 2019.

59. Chelleri C, Guerriero V, Torre M, Brolatti N, Piccolo G, Mattioli G, Boero S, Minetti C, Diana MC. Anterior chest wall deformities in children with neurofibromatosis type 1. Acta Paediatr. 2021 Feb;110(2):594-595.

60. Billar RJ, Manoubi W, Kant SG, Wijnen RMH, Demirdas S, Schnater JM. Association between pectus excavatum and congenital genetic disorders: A systematic review and practical guide for the treating physician. *J Pediatr Surg*. 2021;56(12):2239-2252.

61. Stevenson DA, Zhou H, Ashrafi S, Messiaen LM, Carey JC, D'Astous JL et al. Double inactivation of NF1 in tibial pseudarthrosis. *Am J Hum Genet.* 2006 Jul;79(1):143-8.

62. Margraf RL, VanSant-Webb C, Mao R, Viskochil DH, Carey J, Hanson H, D'Astous J, Grossmann A, Stevenson DA. NF1 Somatic Mutation in Dystrophic Scoliosis. J Mol Neurosci. 2019 May;68(1):11-18.

63. Steinmann K, Kluwe L, Friedrich RE, Mautner VF, Cooper DN, Kehrer-Sawatzki H. Mechanisms of loss of heterozygosity in neurofibromatosis type 1-associated plexiform neurofibromas. J Invest Dermatol. 2009 Mar;129(3):615-21. doi: 10.1038/jid.2008.274. Epub 2008 Sep 18. PMID: 18800150.

64. Wang W, Nyman JS, Ono K, Stevenson DA, Yang X, Elefteriou F. Mice lacking Nf1 in osteochondroprogenitor cells display skeletal dysplasia similar to patients with neurofibromatosis type I. *Hum Mol Genet*. 2011;20(20):3910-3924.

65. Nichols CA, Gibson WJ, Brown MS, Kosmicki JA, Busanovich JP, Wei H, Urbanski LM, Curimjee N, Berger AC, Gao GF, Cherniack AD, Dhe-Paganon S, Paolella BR, Beroukhim R. Loss of heterozygosity of essential genes represents a widespread class of potential cancer vulnerabilities. *Nat Commun*. 2020;11(1):2517.

66. Culling CFA, Allison RT, Barr WT. Cellular Pathology Technique. Four Edition. Butterworth and Co; 214-216.

67. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405-24.