NF1 Truncating Mutations Associated to Aggressive Clinical Phenotype with Elephantiasis Neuromatosa and Solid Malignancies

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Abstract. *Background/Aim: Von Recklinghausen disease is a syndrome characterized by a wide phenotypic variability giving rise to both, cutaneous and visceral benign and malignant neoplasms. The first include cutaneous neurofibromas, subcutaneous and plexiform neurofibromas. The latter can undergo malignant transformation and/or determine elephantiasis neuromatosa. Visceral tumors may include malignant peripheral nerve sheet tumors, gastrointestinal stromal tumors, cerebral gliomas and abdominal neurofibromas. In the present study, the authors discuss the clinical and biomolecular characterization of a cohort of 20 families with a diagnosis of type 1 neurofibromatosis. Patients and Methods: Clinically, the cohort includes three probands with elephantiasis neuromatosa and a peculiarly high incidence of breast and gastrointestinal cancer. Results: Among the 14 NF1 mutations documented, 10 encoding for a truncated protein have been associated to particularly aggressive clinical phenotypes including elephantiasis neuromatosa, malignant peripheral nerve sheet tumors, breast cancer, gastrointestinal stromal tumors. Conclusion: This effect on protein synthesis, rather than the type of NF1 mutation, is the key to the explanation of the genotype-phenotype correlations in the context of neurofibromatosis type 1.*

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Cutaneous or subcutaneous neurofibromas, plexiform neurofibromas, axillary or inguinal freckling, optic gliomas, iris Lisch nodules and multiple café au-lait spots are the main clinical features of NF1 (Neurofibromatosis type 1), also called von Recklinghausen disease or peripheral neurofibromatosis, which is one of the most common autosomal dominant disorders, with nearly 100% penetrance by adulthood (1). Skin lesions, termed cutaneous neurofibromas, derived from skin sensory nerves or single nerve ending such as dermal tumors associated with large nerves, may spread within the dermis and appear as a diffuse mass. Another type of lesion, plexiform neurofibromas (PNFs), usually involves major nerve trunks or nerve plexi; sometimes visible on the surface of the body, but it may be also internal. Lesions visible on the skin surface, may arise from superficial peripheral nerves or represent the superficial extension of a deeper massive plexiform mass (2). While cutaneous neurofibromas are detected in almost all NF1 adult patients, PNFs are present in 30-50% (1, 3) of NF1 patients; a fraction of PNF (10-15%) may transform into malignant peripheral nerve sheet tumors (MPNSTs) (4, 5) and can, though rarely, determine clinical aspects of hypertrophy or a clear-cut elephantiasis neuromatosa (EN) (6). It is known that in the context of NF1 we can find a wide phenotypic variability, both in the same family and among different

families. It is, therefore, possible to find patients with few skin lesions and mild clinical phenotype, along with patients presenting an aggressive clinical phenotype, including gastrointestinal stromal tumors (GISTs), brain tumors (low- and high-grade gliomas), neuroendocrine tumors (somatostatinomas, pheocromocytomas), haematologic tumors (leukemias), breast cancer (7-9) underlying the malignant progression of neurofibromas (5). PNF, when plexiform and deep, can define cases of hypertrophy and/or EN involving the trigeminal nerve, the trunk, and upper and lower extremities (10, 2). In particular, EN is a rare clinical manifestation enclosed in the NF1 phenotype and derives from plexiform neurofibromas of deep nerves associated to hyper-proliferation of the bone and the perineural connective tissue infiltrating adjacent fat and muscles, containing a mixture of Schwann cells, fibroblasts, reticulin, collagen fibers, and a loose mucoid matrix (6). Furthermore lymphedema, in EN, based on a congenital lymphatic disorder, determines secondary adipocyte hyperplasia possibly based on cellular trans-differentiation. In fact, the lymphostasis due to both primary and secondary lymphedema determine the fat cell transformation resulting in hypertrophic adipose tissue and fibrosis (11).

Despite the wide genotypic and phenotypic variability in the context of NF1, just a few genotype-phenotype correlations have been reported. It is known that deletions in *NF1* are associated to more aggressive clinical phenotypes with an earlier onset. *NF1* deletions have been found in association to congenital plexiform neurofibromas and/or appearing in early childhood (<1 year), characterized by cranio-facial dysmorphisms, and by a particularly complex neoplastic spectrum, including solid tumors in various locations exhibiting a higher risk of malignant transformation for neurofibromas (12). There have been no studies investigating on the role of *NF1* truncating mutations in NF1 syndrome cases with EN and/or solid neoplasms. In this study, we have described the clinical and biomolecular characterization of a family cohort with NF1, with particular attention to the phenotype of EN and other solid neoplasms. A secondary aim was the analysis of genotype-phenotype correlations in the assessment of a prognostic role for NF1 truncating mutations in the pathogenesis of particularly aggressive NF1 phenotypes.

Patients and Methods

Patients. Between 1999 and 2013, twenty families clinically-diagnosed with NF1 have been identified in the district of Modena. Reconstruction of family pedigrees of at least three consecutive generations, collection of photographic documentation of cutaneous clinical features of different first, second and third degree relatives, together with evaluation of phenotypic variability (inter- and intra-familial) and type. Fourteen of these families have been clinically and instrumentally followed-up to 15 years.

Germline mutation analysis of NF1 gene. Genomic DNA was extracted from the peripheral blood of patients with NF1 using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA), and stored at –20˚C until use. All of the *NF1* exons were amplified by PCR with intron spanning primers as described by (13, 14) and analyzed with denaturing high-performance liquid chromatography (DHPLC), as described elsewhere (15). For each abnormal elution profile, genomic DNA was directly sequenced in both directions using a CEQ Dye-Terminator Cycle Sequencing Kit (Beckman Coulter Inc., Miami, FL, USA) according to the manufacturer's protocol. Mutations were checked using the Mutalyzer program (https://mutalyzer.nl/).

Results

Clinical characterization. Twenty patients were clinically diagnosed with *NF1*; 14 of them carried an *NF1* germline mutation (8 females and 6 males; mean age=52, 07; range=29-73 years). The tumor spectrum in the NF1 probands included colonic and duodenal adenomatous polyps, two colonic adenocarcinomas, two GISTs, three breast tumors and cerebral glioblastomas. Among affected first-degree relatives, adenomatous polyps, cerebral glioblastoma, colon cancers, breast cancers, pancreatic cancer and kidney cancers were detected. In particular, duodenum cancers (GIST and adenomatous polyps) were diagnosed in four probands, kidney tumors in five first-degree relatives, breast cancer in four first degree relatives and pancreatic cancer in two first degree relatives (Table I). Three probands showed EN and a peculiarly high incidence of breast and gastrointestinal cancer.

Our first patient was a Caucasian female with a case of giant elephantiasis of the arm and numerous cutaneous neurofibromas, some of them pedunculated and of major dimension. The family history highlighted familial segregation with generational anticipation of NF1 phenotype (Figures 1 and 2). The proband underwent surgical enucleation of an arm tumor. Pathology revealed at gross examination a skin flap of 30×10 cm with 4 nodular lesions, respectively of 5, 9, 10 and 11 cm of diameter. The minor lesion was a dermal pedunculated nodule protruding from the skin, while the other three lesions were deeper and subcutaneous. Microscopically all the nodules were composed by small spindle cells with indistinct cell borders, scanty pale cytoplasm and elongated nuclei with a serpentine configuration and pointed ends set in a collagenous stroma. Inflammatory cells, particular mast cells, and small nerve fibres were also present (Figure 3). Positive staining for S-100 protein and CD-34 positivity were also observed. The diagnosis was of multiple neurofibromas. Genetic analysis detected the germline mutation g.116321C>T; c.1318C>T.

Our second patient was a female patient brought to our attention because of a 29-year history of several neurofibromas and multiple *cafè-au-lait* macules. The patient presented giant elephantiasis of the right leg, which started to

Table I. *Clinical and biomolecular features of patients with NF1 syndrome in the district of Modena.*

Patient no.	Gender (age in years)	Germline mutation	Clinical features	NFs and Tumors in the family (age in years)
1.	F (61)	g.116321C>T; c.1318C>T; p.(Arg440*)	EN, NFs (face, trunk, abdomen), >6 CAL, molluscus fibrosum	Grandfather: NFs; Father: >6 CAL, NFs; Son: >6 CAL, NFs; Nephew: >6 CAL
2.	F (29)	g.129042_129043delAG; c.1541_1542del; p.(Gln514Argfs*43)	EN, NFs (abdomen, trunk, giant L4), >6 CAL, two central nervous system hamartomas of the pallidus nucleous, mild lombo-sacral meningocele, scoliosis	
Father	M (56)	g.129042_129043delAG; c.1541_1542del; p.(Gln514Argfs*43)	NFs (trunk), >6 CAL, macrocephaly, hypertelorism	
3.	M (36)	Exon 20 c.3457_3460delCTCA p.(Leu1153Metfs*4)	NFs (abdomen, limbs, face), >6 CAL, axillary freckling, oligophrenia, EN and plexiform neurofibroma of the forehead, hearing loss, orbito-sphenoid dysplasia	Mother: NFs; Daughter: NFs; Aunt: Larynx (68); Cousin: Pancreas (53)
Mother	F (73)	Exon 20 c.3457_3460delCTCA p.(Leu1153Metfs*4)	NFs (abdomen, limbs, face) >6 CAL, axillary freckling	
4.	F (55)	g.139878del; c.2870del; p.(Asn957Ilefs*5)	NFs (trunk, face), >6 CAL, breast cancer	Mother: NFs Cousins: Breast Cancer (34, 44)
5.	M (71)	Exon 20 Del c.3457-3460delCTCA p.(Leu1153Metfs*4)	NFs (trunk, face), >6 CAL, adenocarcinoma of the rectum, GIST, axillary freckling	Mother: renal cell carcinoma (75); Brother: renal cell carcinoma (60)
6.	M (56)	Exon 6 c.750delT p.(Phe250Leufs*31) Lisch nodules, squamocellular	NFs (trunk), >6 CAL, axillary and inguinal freckling, plexiform neurofibroma of the scalp, carcinoma of thyroid with linphonodes and soft tissue metastases	Brothers: NFs, lung (70); Son: NFs; Mother: NFs; Uncles: NFs; Aunt: NFs
7.	F (58)	c.2094-2095delCT	NFs (face, trunk, tail of pancreas), adrenal adenoma, duodenal polyps	Son: NFs, >6 CAL, lipomas, glioblastoma multiforme (26); Son: NFs, > 6 CAL
8	F (40)	c.3916 C>T	NFs, > 6 CAL, breast cancer	Daughter: >3 CAL; Grandfather: NFs
9	M (47)	Exon 4 c.479 G >C p.(Arg160Thr)	NFs (trunk, face), >6 CAL, Lisch nodules, seminoma, adrenal adenoma	Mother: breast cancer (59)
10.	M (50)	Exon 6: large deletion	NFs (face, chest, scalp), >6 CAL, axillary and inguinal freckling, GIST, adenocarcinoma of the colon	Mother: NFs, colon (70); Brother: colon (59); Uncle: NET (72); Aunt: lung (52)
11	F (33)	Intron 46, c.8051-70A>T	NFs (trunk, limbs), >6 CAL, optic glioma axillary freckling	Aunt: lung (72)
12	F (64)	Exon 20 c.2093_2094delCT p.(Pro698Argfs*3)	NFs (trunk, limbs, face), >3 CAL, axillary and inguinal freckling, Breast cancer, multiple lipomas, adrenal adenoma, hepatic hemangioma, inflammatory polyps of the colon	Son: glioblastoma multiforme (30); Son: NFs, >3 CAL

EN: Elephantiasis neuromatosa; NFs: neurofibromas; CAL: *cafè-au-lait* spots; GIST: duodenal gastrointestinal stromal tumors; NET: neuroendocrine tumor.

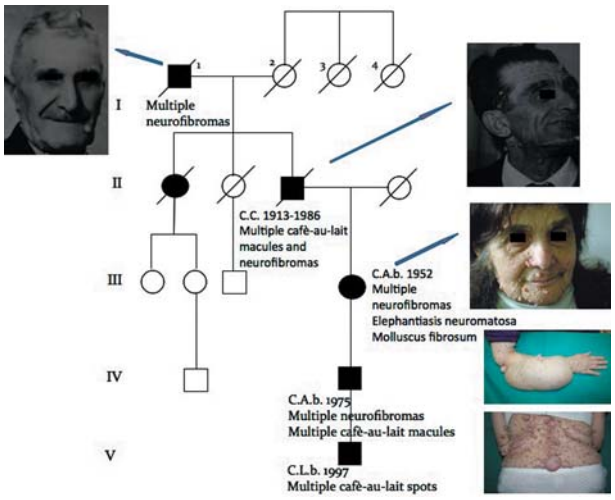

Figure 1. *Family pedigree and clinical features of NF1 proband (proband 1) with EN of the arm cosegregating NF1 germline mutation g.116321C>T; c.1318C>T.*

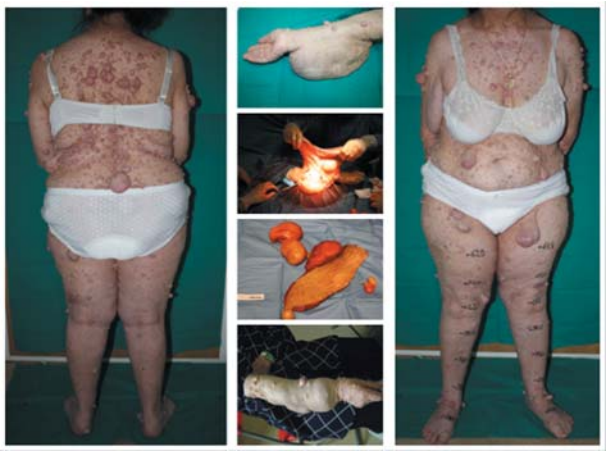

Figure 2. *Clinical features of NF1 proband (proband 1) with EN of the arm.*

grow during late childhood, and accelerated its expansion in the following years (Figure 4). At birth she showed a *café-au-lait* macule on the right thigh. By age 1, she developed a semi-liquid mass at the same site with the aspects of a lymphangioma. Lymphedema of the ipsilateral foot and discrepant leg lengths were noted successively. In the following years, the leg growth was associated to bone proliferation, which required many osteotomies aimed to stop the growth. Two CNS hamartomas of the nucleous pallidus were also identified by MRI. Personal history and 3D-CT revealed a small lombo-sacral meningocele, a giant L4 neurofibroma and significant scoliosis. Her family history was suggestive for NF-1 since her father had macrocephaly, hypertelorism and multiple *cafè-au-lait* macules and neurofibromas. *NF1* germline deletion g.129042_129043 delAG; c.1541_1542del; p.(Gln514Argfs*43) was found both, in the proband and her father.

Patient number 3 was a 36-year-old patient, presenting at birth with giant elephantiasis and plexiform neurofibromas of the forehead, partially covering the left eye. Surgical correction of such a lesion (Figure 5) allowed the patient to partly recover the function of the injured eye. The same patient had also multiple NFs of the abdomen, limbs and face, more than six CALs, oligophrenia, orbital and sphenoid dysplasia and hearing loss. At the age of 36, he underwent surgical amputation of the left leg due to the diagnosis of malignant neurofibromas. His mother and sister had also NFs and CALs; the mother carried the same mutation as the proband, with a truncating effect on protein Exon 20 c.3457_3460delCTCA p.(Leu1153Metfs*4) (Table I).

NF1 gene mutation analysis. The identified constitutional *NF1* gene mutations ranged from single base pair substitutions to gross deletions and microdelesions appeared to be uniformly distributed across the gene. In detail, among the 14 *NF1* mutations, 8 (57,1%) were deletions and 10 (71,4%) led to a truncating effect on the protein (Table I). Two identical mutations (c.3457_3460delCTCA) in exon 20 were associated to different clinical features in two NF1 families unrelated, but with common proclivity to multiple tumors arising in the same patient and with a higher tumor burden per family.

Truncating mutations were mainly distributed among the probands with familial history of NF1 and complex neoplastic spectrum, including, in particular, breast cancer, while just one case appeared as first mutation. Moreover, such mutations with truncating effect were associated to particularly aggressive phenotypes and characterized by generational anticipation for both onset age and aggressiveness of phenotype (probands 1, 2, 3 and 8 of Table I). Three of the above mentioned probands with truncating mutations presented with EN; one case had EN of the arm (patient 1 in Table I) (Figure 2), the second of the lower limb (patient 2 of Table I) (Figure 4), and the third of EN-like lesions constituted by pachydermoceles of the frontal and ocular region (patient 3 of Table II) (Figure 5).

Discussion

Clinical and biomolecular characterization of the family cohort with NF1 diagnosis discussed here showed, along with a wide phenotypic variability, a non-negligible role for EN and other neoplastic manifestations, showing a specific association between these severe clinical cases and

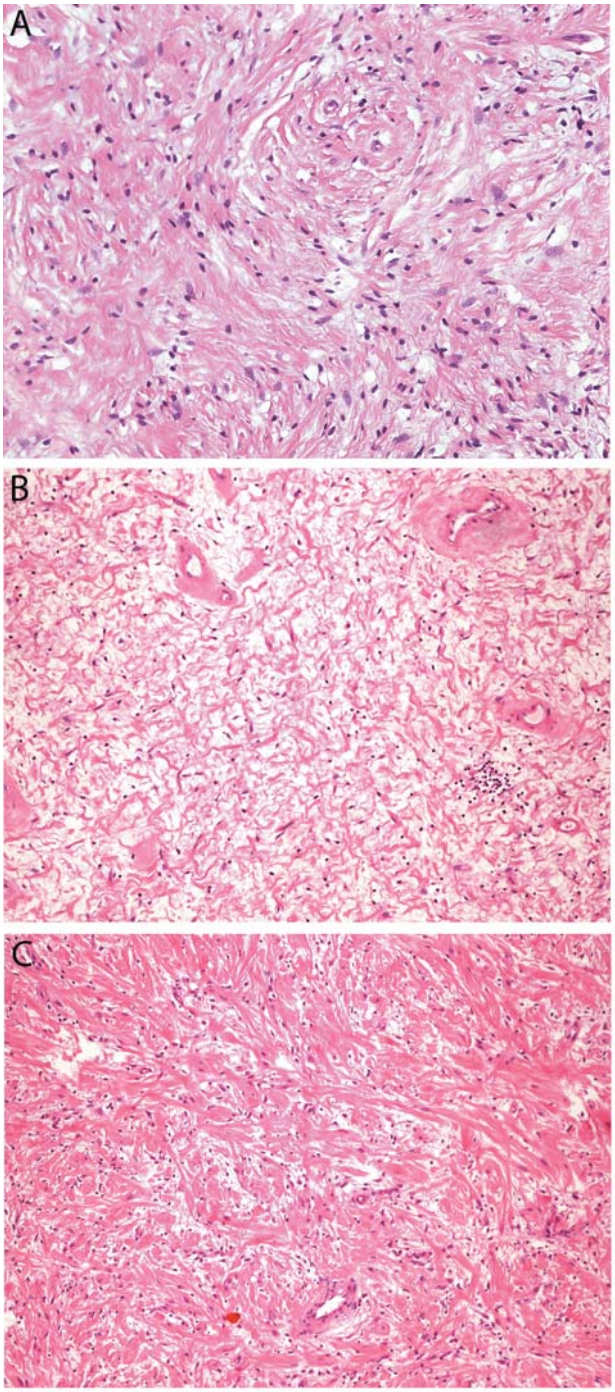

Figure 3. *Representative histological images (H&E) of neurofibromatous lesions involving in the elephantiasis neuromatosa of the arm of proband 1.*

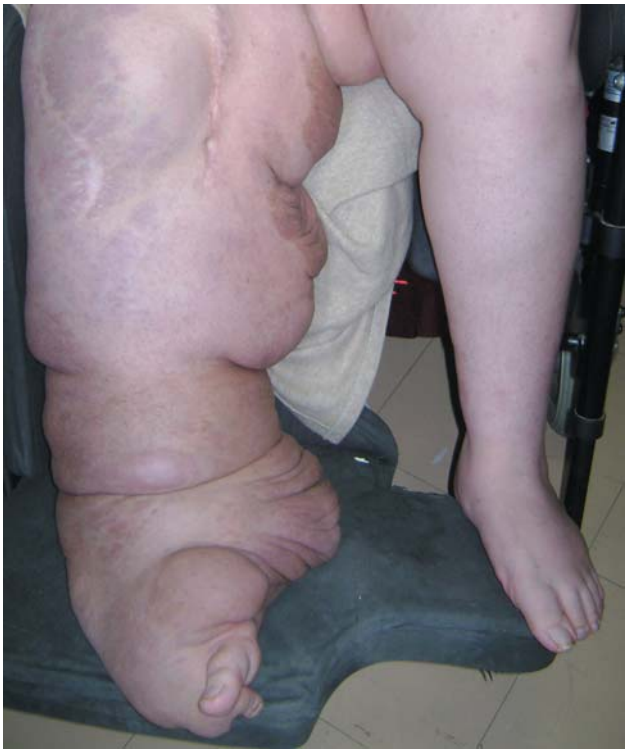

Figure 4. *Clinical aspect of Elephantiasis Neuromatosa of the leg (proband 2).*

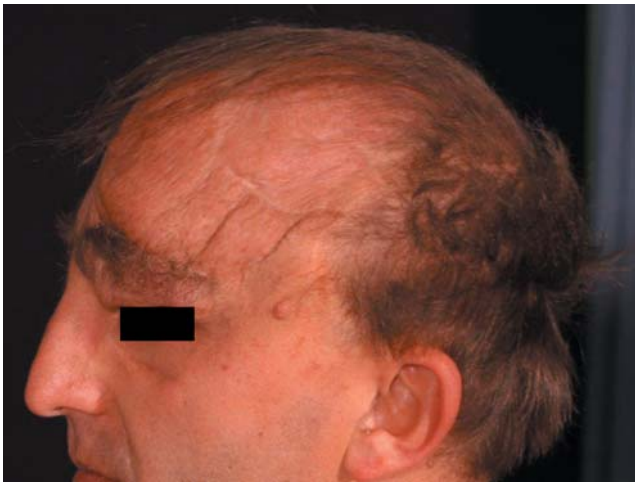

Figure 5. *Clinical aspect of plexiform neurofibroma of the head after the partial surgical resection (proband 3).*

truncating mutations of *NF1*. Less than 50 EN cases are described in the scientific literature; in the majority of the cases genetic characterization was not performed. The first question concerns whether the real incidence of this clinical phenotype could be underestimated. Among the cases already considered, there are no significant differences concerning gender, in most cases, however, they are segregated following the maternal part of the family. In a

Table II. *Elephantiasis neuromatosa as clinical manifestation enclosed in the NF1 phenotype.*

Gender - Age/ Age of onset	Family history	Proband affected regions and clinical features	Reference
M - 20 years/ At birth	Grandmother (2000 minute cutaneous tumours and scattered subcutaneous swellings), mother (achondroplasia).	Gross elephantiasis neuromatosa of the left limb. Subcutaneous tumors scattered all over the body; a pachidermatocele of the occipital scalp; cutaneous freckles. Bone development increasing.	Spittel RL and Fernando SE, 1929.
M - 40 years/ At birth	Mother affected by type I neurofibromatosis.	Elephantiasis neuromatosa of the neck. Deformity of the left clavicle, left leg and cervical column.	Westcott RJ and Ackerman LV, 1947.
M - 22 years/ Childhood	There was no particular familial history of neurofibromatosis.	Elephantiasis neuromatosa of extensively involved the chest wall and the axilla.	
F - 42 years/ -	No family history for the type I neurofibromatosis.	Soft irregular bluish end mottled mass involving the distal two-thirds of the right lower extremity.	Lenson N, 1956.
M - 16 years/ Childbirth	Not reported.	Elephantiasis neuromatosa of the penis. Syringomyelia associated with a cervical ependymoma. Facial paralysis on the left side and paralysis of the left eye muscle. Multiple schwannomas involving some cranial nerves.	Fethiere W, Carter HW and Sturim HS, 1974.
M - 11 years/ 3 years	Not reported.	Two cases of elephantiasis neuromatosa respectively of the left thigh and right leg, overgrowth of abnormal bones with subperiosteal haemorrhage.	Yaghmai I and Tafazoli M, 1977.
F - 9 years/ 2 years	Not reported.	Subsequent rapid ossification resulted in residual diaphyseal widening after minor trauma.	
M - 4 years/ -	Eleven of his relatives have neurofibromatosis.	Elephantiasis neuromatosa involving the right lower extremity. Numerous *cafè-au-lait* spots on the lower abdomen and groin.	Sty JR, Starshak RJ and Woods GA, 1981.
F - 18 years/ At birth	No family history for the type I neurofibromatosis.	Only one large *cafè-au-lait* spots on the right hallux. Soft tissue hypertrophy with massive enlargement of both proximal and distal phalanges.	Harris WC Jr, Alpert WJ and Marcinko DE, 1982.
F - 17 years/ Childhood	There was no predisposition to either familial or hereditary disorders.	Elephantiasis neuromatosa of the right gluteal sulcus, coexistent with lipomatosis. Absence of skeletal lesion.	Holck S, Medgyesi S, Darre E and Lassen M, 1984.
F - 30 years/ At birth	Not reported.	Elephantiasis neuromatosa of the left lower limb maximal in the calf. Dysplastic bones of the left hemipelvis and leg.	Birch PD and Davies AM, 1988.
F - 52 years/ Childhood	The patient denied any manifestations of neurofibro-matosis in her family.	Symmetric elephantiasis neuromatosa of the trunk. Hypovolemic shock at the age of 32 years.	Hertzanu Y, Hirsch M, Peiser J and Avinoach I, 1989.
M - Few months/ At birth	No family history of congenital glaucoma or buphthalmos.	Buphthalmos and regional gigantism with evolution in neurofibromatous elephantiasis. A swelling of the right upper lid and preauricular region. Hypertrophy of the soft periorbital tissues. Corneal diameter 13 mm, rubeosis iridis, ectropion uveae,	Bardelli AM and Hadjistilianou T, 1989.
M - 13 months/ At birth	Not reported.	increased optic nerve cupping. Buphthalmos, an enlarged optic canal, a fibrous dysplasia of the greater wing of s phenoid bone. Longer right fibula and a	
F - 11 years/ At birth	Not reported.	slight deformity of the vertebrae of the lumbal tract. Facial elephantiasis without buphthalmos. Greater wing of sphenoid bone; right optic foramen larger than the left. Iris nodules, ectropion uveae, increased optic nerve cupping. Small neurofibromas of the zygomatic and temporal region.	

Table II. *Continued*

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Gender - Age/ Age of onset	Family history	Proband affected regions and clinical features	Reference
F - 34 years/ Since 8 years	His mother, grandmother, sister, nephews had neurofibromatosis.	Elephantiasis neuromatosa involving the right lower limb and pelvis, leading to a right hip disarticulation. Large neurofibroma of the sciatic nerve.	Kuo LA and Kuo RS, 1990.
F - 22 years/ At birth	No family history for the type I neurofibromatosis.	Enlargement, overgrowth and elongation of all the bones of right the foot.	Roy SM and Ghosh AK, 1992.
M - 21 years/ At birth	No cases reported in this family.	Gigantism and elongation of all the bones of the right foot.	
M - 18 years/ Childhood	His father had elephantiasis neuromatosa.	Gigantism and elongation of all the bones of the right foot.	
M - 6 years/ At birth	No family history for the type I neurofibromatosis.	Enlargement mainly of the forefoot and the swelling was more prominent between the great and 2nd toe.	
M - 3 months/ At birth	Family history did not reveal occurrence of similar illness	Congenital plexiform neurofibroma involving neck with elephantiasis in any of members.neuromatosa with sarcomatous nodule. The skin covering the tumor was hairy, redundant and dark.	Kokandkar HR, Vyas AS, Kumbhakarna NR and Totala RJ, 1994.
F - 33 years/ 6 years	Family history of neurofibro-matosis.	Elephantiasis neuromatosa of the right leg. Severe dysaesthesia in the right lower leg not confined to a single nerve.	Münte TF, Matzke M, Johannes S, Dietrich B and Dengler R, 1996.
M - 43 years/ Childhood	Not reported.	Gross elephantiasis neuromatosa of the left leg. Large synovial cyst arising from the synovium of the patello-femoral joint.	Stevens KJ, Ludman CN, Sully L and Preston BJ, 1998.
M - 20 years/ 6 years old	No family history for the type I neurofibromatosis.	Elephantiasis neuromatosa with Becker's melanosis. Hairly and brown-black hyperpigmented patches on left shoulder, left upper back and left arm. Lisch nodules.	Akyol M, Ozçelik S, Marufihah M and Elagöz S, 1999.
M - 35 years/ -	Not reported.	Elephantiasis neuromatosa of the right thigh and sacral region. A soft tissue mass and enlargement of the right upper leg.	Lorberboym M, Trejo L and Lampl Y, 2000.
F - 13 years/ At birth	Her mother had type I neurofibromatosis.	Elephantiasis of the left leg with recurrent massive subperiosteal haematoma.	Steenbrugge F, Poffyn B, Uyttendaele D et al., 2001.
F - 41 years/ Childhood	Not reported.	Elephantiasis neuromatosa of the right leg. Optic chiasm glioma. Right tibia and fibula marrow and cortices hypertrophy.	Hourani R, Rizk T, Kung S and Boudghène F, 2006.
F - 14 years/ At birth	Not reported.	Elephantiasis neuromatosa involving the right lower limb. Anaemia and hepatitis B.	Martínez-García S, Vera-Casaño A, Eloy-García Carrasco C et al., 2008.
M - 56 years/ Childhood	There was no particular familial history of neurofibromatosis.	A huge mass of elephantiasis neuromatosa in the right leg.	Hoshi M, Ieguchi M, Taguchi S and Yamasaki S, 2009.
M - 15 years/ Childhood	No family history for the type I neurofibromatosis in first-degree relatives.	Elephantiasis neuromatosa of the right leg. Osseous abnormalities included thinning of bones, erosion of distal articular surfaces and periosteal dysplasia.	Bano S, Prasad A, Yadav SN et al., 2010.

M: Male; F: female.

great majority the clinical expression was congenital, since it was diagnosed at birth or during the first decade of life (Table II). From the clinical definition and pathogenesis of plexiform neurofibromas point of view, the overgrowth of connective tissue may be limited to a single superficial nerve or plexus; when the spread is limited to epithelial tissues, clinical appearance consists of soft fibrous tumors, named molluscum fibrosum or pachidermocele (as in our patient 3), likely histologically corresponding to mixoglioma gelatiniforme, a plexiform neurofibroma variant lacking muscle and Schwann cells, not associated to an increase in the bony development (16, 17). The term EN, referring to the cutaneous-confined phenotype, might be inappropriate, since a distinct superficial dysplastic skin alterations known as

pachidermocele or dermatholysis, histologically corresponding to a gelatiniform mixed glioma, must be distinguished from EN in patients affected by NF1. Our data show that a hyperproliferative process involving the soft tissues, bones and lymphatic system beyond the peri-neural connective can be responsible of EN in NF1. The lymphostasis and the following lymphedema trigger an adipocyte metaplasia whereas the chronic hyperemia produces bone hypertrophy. Lymphoscyintigraphy and MRI can be an efficacious tool in the diagnosis and clinical characterization of early onset cutaneous, subcutaneous and skeletal anomalies, allowing for detection of details that would not be visible with traditional radiograms and enabling an in-depth anatomical study together with a pre-operative assessment. In fact, an appropriate clinical and instrumental management, including the evaluation of the functionality of the vascular and lymphatic system, is critical for classifying EN in its complex pathogenesis and in the selection of those cases, that have to be surgically treated with liposculpture and/or surgical enucleation of neurofibromas. Timing and approach type, based on specific *NF1* mutations predictive of clinical aggressiveness, might be organized and modulated. However, it is already well-known that the effective number of genotype-phenotype correlations is exceedingly low; however, one of them is related to the role of large *NF1* deletions. Truncating mutations produce a stop-codon which generate a short protein with a reduced function. From a clinical point of view, the molecular diagnosis of a large *NF1* deletion is important because it is frequently associated with severe clinical manifestations including an increased risk of MPNSTs as compared with the general NF1 population (18-20). It is widely assumed that genotype-phenotype correlations in patients with large *NF1* deletions are likely to be influenced by both the number and type of deleted genes.

Concerning *NF1*-associated neoplasms, *NF1* patients are at increased risk of several types of neoplasia (7, 21, 22). There are data suggesting an increased risk of different malignancies, including breast cancer and leukaemia, among patients with NF1 (23, 24). Sharif *et al*. (24) reported an increased risk of breast cancer among female patients with NF1. The authors analyzed a British case series of 304 females with NF1, and found that women with NF1 had a 5-fold increased risk of being diagnosed with breast cancer, being the most common type of breast cancer an infiltrating ductal carcinoma. In particular, the increased risk appeared to be specific to women under 50 years of age. Similarly, Seminog *et al*. (25) reported a three-fold risk for breast cancer in women under 50 years with NF1. A different study that looked at mortality in NF1, found that women with NF1 were 3.5-times more likely to die from breast cancer than women in the general population (PMR=3.5; 95%CI 1.3–7.7) (9). There have been numerous case reports of patients with NF1 who presented ductal type breast carcinomas, including a male patient who was diagnosed with bilateral ductal carcinoma at age 18 (26).

We reported an equally high risk of breast cancer among *NF1* female gene-carriers and their family members, and agree on the opportunity of offering a screening program that includes early and annual clinical and radiographic screening for breast cancer in those patients with clinical and biomolecular diagnosis of NF1 (27).

Although the phenomenon of generational anticipation of NF1 syndrome was not previously discussed in the scientific literature, this feature is certainly present in our experience, both in families and probands with EN carrying *NF1* germline mutation, as well as in those without EN. In particular, as in the case discussed above (Figure 1), the anticipation phenomenon regarded both, aggressiveness and onset age of the cutaneous phenotype. Recent evidence has shed light on the role of modifier genes and of environmental factors in the pathogenesis of intra- and inter- familial phenotypic variability. It clearly appears that the clinical expression of NF1 tends to be similar in close relatives and will decrease with the degree of relatedness (28). In particular, studies on homozygote twins showed that genetic factors determined the onset of particular phenotypic features and in one case, twins with markedly different NF1 phenotype (only one with NF1 features) carried a post-zygotic *NF1* gene mutation, identified in the affected twin who presented a somatic NF1 mosaicism (29). It is possible that, similarly to other genetic diseases, NF1 can be affected by the accumulation of progressive mutations that, in case of familial segregation, can determine an anticipation of onset age and an increased aggressiveness of the phenotype throughout the generations.

Concerning potential modifier genes, it is known that an early reduction in mismatch repair capacity can lead to an accumulation of second hits in NF1; so it is possible to hypothesize that overlapping molecular pathways might underlie a greater susceptibility to the acquisition of second mutations, both germline and somatic, that are responsible for phenotypic characterization.

Previous studies (30) were not able to find any clear relationships between a *NF1* mutation and distinct clinical features and some authors hypothesized that the phenotypic differences in NF1 patients are more likely to be caused by mechanisms such as a second hit, modifying genes and stochastic events (31).

While discussing the somatic mutational spectrum of NF1 associated tumors Laycock-van Spyk *et al*. (32) reported that about 75% (191/254) of the somatic mutations associated with *NF1* tumors comprise mutations that are predicted to give rise to truncated proteins and 173 of these truncations arise form deletion, nonsense mutation or frameshift events. In particular, the authors highlighted that the high proportion of truncating mutations were involved in the somatic

inactivation of the *NF1* gene in cutaneous neurofibromas. An Italian study on constitutional *NF1* mutations by De Luca *et al*. reported 61 out of 75 (81%) truncating mutations (33).

Biomolecular evidence regarding PNFs associated to EN and/or MPNST suggest an equally critical role of *NF1* germline aberrations, leading to the truncating protein effect. The biomolecular study confirmed a pathogenetic role for big deletions in the genesis of particularly aggressive and complex clinical phenotypes, highlighting an association between NF1 truncating mutations and EN, MPNST and NF1-associated malignancies. Future biomolecular studies shall be based not only on the analysis of *NF1* mutation types, but also on their direct effect on the protein synthesis, and on a wider case series including patients with EN- and other NF1-associated tumors. Therefore, it will be possible to clarify the possible phenotypic correlations of NF1 germline mutations with truncating effect and their potential consequences in terms of screening and follow-up planning.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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