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# Clinical trial with Imatinib Mesylate for plexiform neurofibromas in Neurofibromatosis type 1 patients

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***SUMMARY***

## ABSTRACT

***Background:*** Neurofibromatosis type 1 related-plexiform neurofibromas (PNFs) are invasive and growing tumours, with substantial clinical consequences and with a potential risk of transformation into malignant peripheral nerve sheath tumours (MPNSTs). On the basis of recent studies, a clinical trial to test the hypothesis that inhibition of c-kit signalling pathways by Imatinib Mesylate (IM) results in objective reduction and/or inhibition of the growth of PNF was performed. Furthermore, circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) were evaluated as target and response indicator biological markers for the tyrosine kinase inhibitor treatment.

***Methods:*** 7 patients (aged 5-35 years) with genetically NF1 confirmed diagnosis and suffering from extensive and infiltrative mainly paraspinal located PNFs were enrolled in the study. The trial consisted in oral administration of IM at the dose of 220 mg/m2 twice a day for children and 400 mg twice a day for adults, for 12 months, unless severe toxicity. Immunohistochemistry was performed for confirmation of c-Kit and PDGF-β expression tumour biopsies in all PNFs. Every patient was followed longitudinally 12 months after drug discontinuation. Serum CECs and EPCs were evaluated at baseline and regularly during the treatment.

***Results:*** Only two patients completed the 12-month treatment with an adequate dosage; 1 patient required a lower dosage due to renal failure. Two patients withdrew from the study prematurely because of severe side effects (gastric bleeding and arthrosynovitis respectively), and two others because of evidence of non PNFs additional symptomatic growing lesions. All the patients have a sustained stabilization of PNFs volume progression rate (range 0.8 - 4.5%) over time during the follow-up (range 2.5 – 4.8%). The investigation

of angiogenic factors, revealed higher levels of viable and apoptotic CECs (p=.012 and p=.038 respectively), CD109+ CECs (p=.002) and VEGFR2+(p=.007) at baseline compared to healthy controls. During the treatment, a close to significance reduction of CD109+ CECs (p= .052), CD140b+ PPC (p= .052), and vital CECs (p= .072) were observed. No relationship between angiogenic factors and clinical or neuroradiological severity and evolution was noted during IM treatment (p=.470 and p=.510 respectively).

***Conclusions****:* Patients with extensive and complex PNFs with a primary paraspinal location had not an objective response to IM treatment; however, disease stability lasting 12 months after drug discontinuation was observed in all patients. CECs and EPCs seem to be indicators of anti-angiogenetic Imatinib Mesylate activity rather than useful biomarkers for predicting response or forecasting progression. A better knowledge of natural history of PNFSs in NF1 is needed before performing new clinical trial.

# INTRODUCTION

Neurofibromatosis type 1 (NF1) is the most common dominantly inherited genetic disorder affecting about one in 2500-3000 people worldwide, caused by loss-of-function mutations of the tumour-suppressor gene *NF1*, located on chromosome 17q11, which encodes Neurofibromin, a negative regulator of RAS proteins. It is a complex, childhood-onset, chronic and often progressive, multisystem disorder that requires lifelong, highly specialized care and support.[1]

The disorder is associated with a broad spectrum of dermatological, neurological, cardio-vascular, gastrointestinal, orthopaedic complications. In addition, affected individuals are prone to develop benign and malignant tumours of the central and peripheral nervous system, and malignant diseases affecting other parts of the body.[1-3]

NF1 represents a simply determined Mendelian disorder with a complete penetrance; it is however characterized by age-dependence and highly intra- and interfamilial variable expressivity in both the number of the major features and the occurrence of complications, resulting unpredictable prognosis and evolution.[1]

### *1.1.1 NF1: diagnostic criteria*

Neurofibromatosis type 1 diagnosis is currently still based on the clinical criteria encoded by National Institute of Health (NIH) Consensus Conference statement organised in 1988 in the United States.[4]

The diagnosis is made in an individual with any 2 of the following clinical features:

- Six or more café-au-lait macules with a 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 pathway glioma (OPG);
- Two or more Lisch nodules (iris hamartomas);
- A distinctive osseous lesion such as sphenoid dysplasia or thinning of long bone cortex with or without pseudoarthrosis;
- A first-degree relative (parent, sibling or offspring) with neurofibromatosis 1 by above criteria.

The onset of many features of NF1 is age dependent. It has been observed that about 95% of neurofibromatosis type 1 patients meet the diagnostic criteria by the age of eight years.[5]

The first of the NIH cutaneous clinical criteria is the presence of six or more café-au-lait spots. The *café-au-lait spots* arise from a defect of skin melanocytes and they are present from birth, but become more evident during time: they can multiply until school age, usually stop multiplying during pre-puberty or puberty, and may even decrease in number during adulthood.[6] Clinical experience has shown that number, diameter and shape of these cutaneous lesions are not related with the severity of the clinical expression of the disease.[6]

*Freckling* in the axillary or inguinal region do not differ histologically from café-au-lait spots: they should therefore not be considered as a separate criterion but as "small café-au-lait spots" that appear later in childhood (often after the age of 4–5 years) and have a particular presentation possibly because of their anatomic site.[6]

*Neurofibromas* are benign tumour consisting of a set of heterogeneous cells such as Schwann cells, fibroblasts, perineural-like cells and isolated nerve fibres. They have two

possible clinical forms: protuberant or pedunculated flesh-coloured papules or nodules that are typically soft on palpation, or "button hole-shaped" soft subcutaneous nodules surrounded by a dermal fibrous labrum. They usually begin to develop at about the time of puberty; this clinical criterion is therefore clearly not helpful for an early diagnosis.[7]

*Plexiform neurofibromas* (PNFs) are congenital lesions characterized by a complex microenvironment composed on Schwann cells, fibroblast, perineural cells, mast cells, secreted collagen and blood vessels. Their growth rate is variable, erratic and unpredictable. They can involve different parts of the body and may infiltrate, displace or compress surrounding structures; they are associated to a lifetime risk of malignant progression to MPNSTs in 8-13%. PNFs may be characterised by overlying hyperpigmentation or hypertrichosis.[8]

Among the extra-cutaneous signs of NF1, only Lisch nodules, optic pathway gliomas and bone anomalies are included in the NIH criteria.

*Lisch nodules* are benign melanocytic hamartomas, that can be detected by means of a slit-lamp. Over 90% of adults affected by NF1 presents Lisch nodules but it seems that they are often undetectable in early childhood.[9]

*Optic pahway glioma* (OPG) is the most common tumour in Neurofibromatosis type 1 with a prevalence of 15-20%, occurring predominantly in early childhood; 6 years and younger children with NF1 are at greatest risk for development of OPG.[10] The NF1 associated OPGs are classified by World Health Organization (WHO) as a grade I astrocytoma (pilocytic astrocytoma, PA); some tumours lack classic features of PA and may be classified as WHO grade II.[11] These tumours may arise or progress later in childhood or adulthood and that visual loss is more likely in children whose lesions involve the posterior optic pathway.[11]

Finally, *bone anomalies* are congenital focal osteopathies, which may classically but rarely involve the sphenoid wing (3–7%) and long bones (most frequently the tibia and fibula, 1–4%), or more frequently the spine, with dystrophic scoliosis and kyphosis.[12]

### *1.1.2 NF1: minor clinical signs*

Over the last 20 years, medical understanding of NF1 has naturally improved, and a series of other clinical signs not included in the NIH criteria were reported.

These clinical clues include:

- cutaneous signs: anaemic nevi, juvenile xanthogranulomas, mixed vascular hamartomas and cherry angiomas, hypochromic macules, "soft touch" skin;
- ocular features: choroidal hamartomas;
- neurological clues: unidentified bright objects, a typical neuropsychological phenotype, macrocrania, seizures;
- others: short stature, precocious puberty, deficient bone mineralisation, Noonan like facial traits.

The established diagnostic criteria are very specific, but they are not so sensitive because some of them are not present in infancy. On the other hand, some of the clinical findings above, are not so specific as they can be associated with other partially overlapping disorders, but they could make up the diagnostic path more sensitive and precocious.[7,13,14]

### *1.1.3 NF1: complications*

Complications are of variable severity and frequency and are carefully investigated in patients with the disease.

Affecting different organs and tissues, the condition need a multidisciplinary approach and periodic follow-up.

- oncological: NF1 patients are prone to develop benign and malignant tumours of the central and peripheral nervous system, and malignant diseases affecting other parts of the body. Many tumours in patients with germline NF1 mutations are neural crest cell-derived tumours (pheochromocytomas, neurofibromas, MPNSTs, glomus tumour) or neuroepithelial cell-derived tumours (pilocytic astrocytomas). Non-neural-crest-related cells are also predisposed to tumorigenesis (JMML and rhabdomyosarcoma);[15]
- orthopaedic: subjects with NF1 can develop skeletal abnormalities, including osteopenia, scoliosis, low bone-mineral densities, chest deformities, non ossificans fibroma;[14]
- cardiovascular: individuals with neurofibromatosis type 1 can develop various cardiovascular abnormalities, ranging from congenital heart disease (pulmonary artery stenosis) to vasculopathy (including renal and cerebral artery stenosis, aortic coarctation, and arterovenous malformations) and hypertension;[16]
- neurological: neuropsychological deficits are common manifestations of NF1. Learning difficulties can include visuospatial and visuo-motor deficits, language disorders, and fine and gross motor deficiencies. Furthermore, attention-deficit

hyperactivity disorder, autism spectrum disorders, behavioural abnormalities, and psychosocial issues are prevalent in this population.[17]

## PATHOGENESIS

Neurofibromatosis type 1 (NF1) is caused by loss-of-function mutations of the tumour-suppressor gene *NF1*, located on chromosome 17q11, which encodes Neurofibromin, a negative regulator of RAS proteins. All individuals with NF1 are born with 1 functional and 1 non-functional (mutated) copy of the *NF1* gene in every cell in their body.[18] Approximately half of all NF1 cases are diagnosed without a known family history and are thought to represent new mutations.[18]

Genetic testing in NF1 is challenging because of the large number of possible mutations in the large gene. Linkage analysis can be offered but is not helpful for sporadically affected individuals. Use of a set of complementary techniques, including both DNA and RNA studies, permits detection of 95% of mutations in patients who fulfil diagnostic criteria.[19]

### *1.2.1 NF1 gene*

The disease-causative *NF1* gene spans 350 kb of genomic DNA and contains 61 exons, including 4 alternative spliced exons, and which is transcribed into a 12-kb mRNA containing an open reading frame of 8454 nucleotides.

The *NF1* gene has a TATA-less promoter with a classic CpG-island that is normally unmethylated, and a 454 bp 5'untranslated region (UTR), that exhibits a high degree of sequence conservation with *NF1* genes found in many other organisms. While no *NF1*-causative mutations have been identified within the promoter or 5' UTR, there are a number of associated polymorphism and rare variants.[20-22]

The terminal 3'-UTR is large (3.5 kb) and contains two polyadenylation with a high level of sequence conservation, indicating its possible functional importance either for regulation

mRNA stability or for controlling translational efficiency of the gene.

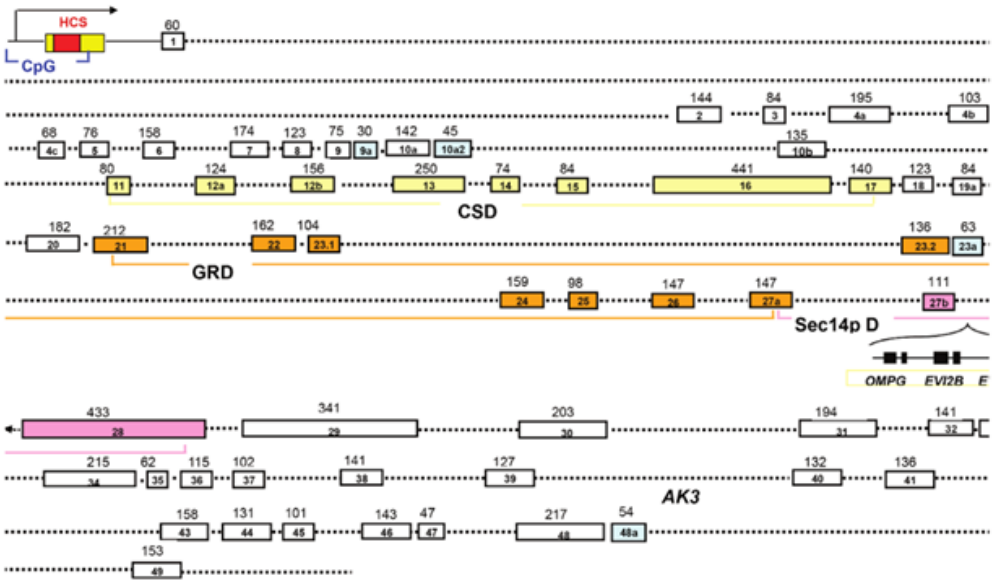
The central portion *(NF1-GRD)*, extended between exons 21 and 27a, constitutes 13% of the entire coding sequence and carries a negative regulation on Ras activation and then on cell growth.

Furthermore, the *NF1* gene has two large introns (1-27b), of which intron 27b contains three small unrelated tumour-suppressor genes (*OMGP*, *EVI2B* e *EVI2A*), transcribed in the reverse orientation to the *NF1* gene. The possible role of these genes, whether individually or together, is to act as natural antisense interfering with RNA processing its transduction.[20,22]

The gene has four alternative spliced exons (9a, 10a-2, 23a e 48a); in each case the inclusion of any one of these exons doesn't disrupt the overall reading frame. A number of different NF1 transcripts have been found to be differentially expressed in various tissues in normal individual. In addition to the original reported 13 kb isoform (type I o GRD I), the ubiquitously expressed type II *NF1* transcript was reported. GRD II includes the alternative spliced exon 23a, that results in a 63-bp in-frame insertion in the GAP-related domain of neurofibromin; the resultant type II neurofibromin, with an additional 21 aminoacid, exhibits a significant reduced GAP activity but conversely demonstrates and increased affinity for Ras in comparison to type 1 neurofibromin that lacks exon 23a.[23]

Another alternative spliced transcript (GRD III) that includes exon 48b, resulting in a 54-bp-inframe insertion is abundantly expressed in muscle and its function is not yet been clarified. Further isoform, containing exon 9a with an additional 30 bp, is highly expressed in central nervous system and possible correlation with neuronal development was

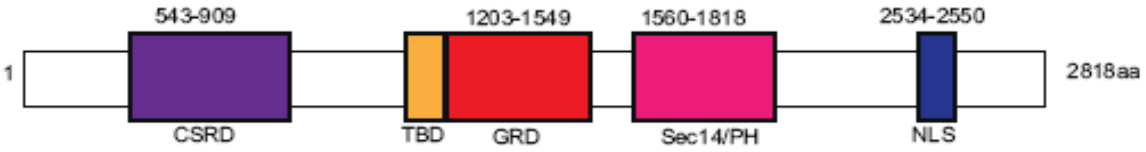
suggested.[20,22,23]




*Schematic representation of NF1 gene*

### *1.2.2 Neurofibromin*

Neurofibromin is a 2818 aminoacid protein that is ubiquitously expressed, with its highest levels present in cells in the CNS, where it is often found in association with tubulin. Although the protein levels differ in different tissues and in developmental or functional states.[24]




*Schematic representation of Neurofibromin*

Neurofibromin localization is predominantly cytoplasmatic but also observed in the nucleus through a site encoded by exon 43 (nuclear localization signal-NLS); it has been suggested that the transport of the protein inside the nucleus represents a mechanism of neurofibromin function regulation. In fact over the past few years it has been shown that many tumour

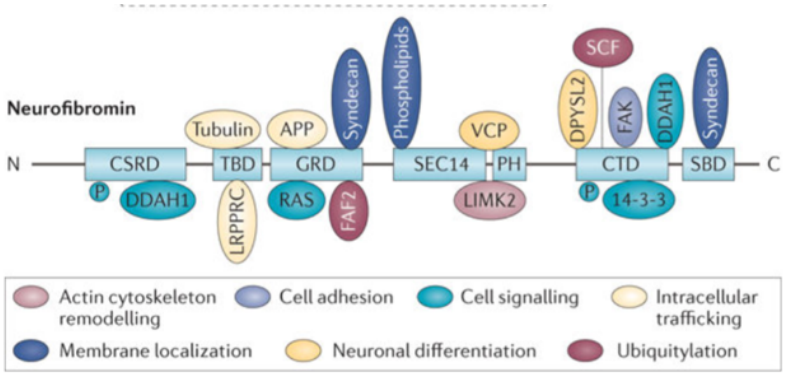
suppressor genes shuttle between the nucleus and the cytoplasm. This type of dynamic intracellular movement regulates not only protein localization, but also protein function [25]

Neurofibromin is a member of the large family of evolutionarily proteins, the Ras-GTPase activating protein (GAP)- related proteins, with the most highly conserved region of the protein being the centrally located GAP-related domain (GRD) encoded by exons 21-27a of NF1 gene.

A second functional domain is encoded by exons 11-17 and it's a site of many missense and inframe mutations. This region coincides with a *cysteine-serine rich domain* (CSRD) and it's a potential site of recognition for the cAMP-dependent protein kinase A (PKA) as well as of interaction with microtubules.[26,27]

Another region Sec14/PH (*Sec14p homology/ pleckstrin homology domains*) seems to interact with LIM domain kinase 2 (LIMK2) and thereby inhibits activation of LIMK2 by RHO-associated protein kinase, which results in modulation of the cytoskeleton.[28]

Due to the complexity of its functional domains, neurofibromin is known to be associated with a large number of proteins, including tubulin, kinesin, protein Kinase A and C, caveolin, cytokeratin intermediate filaments and amyloid precursor protein. This diversity of protein associations does emphasise the many function of the proteins including modulation of cellular trafficking, neuronal differentiation, membrane localization, actin cytoskeleton remodelling, ubiquitylation, cell adhesion, and cell signalling through RAS pathway.[20]

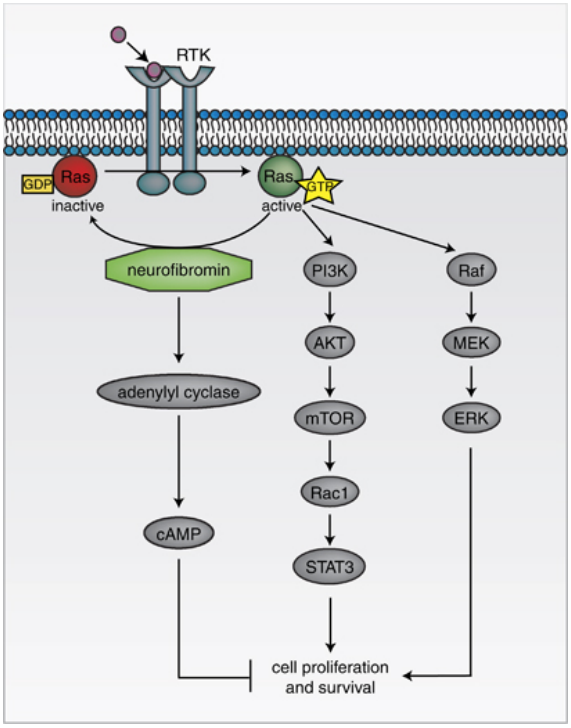



*Schematic representation of Neurofibromin multiple functions*

The most fully characterized function of Neurofibromin is its role in tightly regulation cellular levels of activated Ras protein. All Ras proteins exist in two cellular state, the majority being found in their inactive GDP-bound form, with only a very small fraction present in their metabolically active GTP-bound form. Only in this active state Ras proteins are able to up-regulated the many downstream effector proteins that form part of the large complex Ras/Raf/MAPK signaling pathway. [20 ,29]

The known role of neurofibromin is to down-regulate activated GTP-bound Ras proteins by markedly stimulating the low intrinsic GTPase-activity of the Ras proteins themselves, thus resulting in the rapid conversion of active Ras-GTP into its inactive Ras-GDP state. Hence, any loss of neurofibromin functionally, due to inactivating mutations of the *NF1* gene, will result in sustained cellular levels of active Ras-GTP, leading to prolonged activation of all components of the Ras/Raf/MAPK signaling pathway, along with all its many downstream effector components. The overall result of such activation being loss of growth control and increased cell proliferation. [29]

Furthermore, the active Ras-GTP state leads the transduction of the PI3K/AKT/mTOR pathway, another complex signaling network that regulates cell growth and proliferation.[30]




*Ras and mTor pathways*

## TUMORIGENESIS

Recent studies have permitted the identification of several complementary mechanisms involved in tumours development and progression, providing new targets for pharmacological treatment.

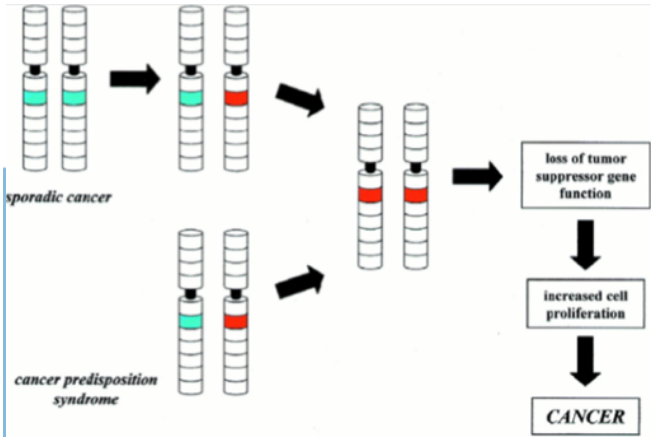
### *1.3.1 Two Hit hypotesis*

Cancer represents the transformation of a cell whose growth is normally tightly controlled into one that is no longer under strict regulation, allowing the cell to multiply uncontrollably and even metastasize. This dramatic alteration in cellular control arises as a consequence of the accumulation of genetic and epigenetic changes: activated oncogenes speed up cell growth through the acquisition of gain-of-function mutations, whereas tumour-suppressor genes (TSGs) promote progression by acquiring loss-of-function mutations. TSGs typically encode proteins involved in growth regulation, apoptosis initiation, cellular adhesion and DNA repair. In accordance with Knudson's two-hit hypothesis, both alleles of a TSG must be inactivated for cellular transformation to occur.[31]

In cancer predisposition syndromes like Neurofibromatosis type 1, patients inherit a germline mutation in one TSG allele; any tumours that arise will have acquired a second somatic 'hit' that inactivates the normal NF1 allele, resulting in a complete loss of functional neurofibromin. A double hit (NF1-/-) is critical for NF1 tumorigenesis occurrence.

At somatic level, the inactivation of the wild type allele may occur through relatively subtle lesions that affect just a few DNA bases, or may involve large genomic changes that affect large chromosomal regions, or even the entire chromosome 17. A recent review concluded

that NF1-associated tumour types display a considerable degree of variation in terms of the level of loss of heterozigosity (LOH) detected. For example, MPNSTs manifest increased levels of deletion-based LOH, whereas cutaneous neurofibromas appear to be associated with a localized deletion of the *NF1* gene through mitotic recombination. In MPNSTs, additional mutations at different gene loci are almost certainly involved in the progression of the tumour.[32]




*The Two-Hit hypotesis*

### *1.3.2 Modifier Genes*

Although the biallelic inactivation of the *NF1* gene described above is the basis for pathogenic process for tumour development, this cannot explain the wide clinical and histopathological inter and intrafamilial variability.

Since their initial description in 1992, many studies have reported a more severe clinical phenotype in patients carrying genomic *NF1* deletions in comparison with patients with intragenic mutations. It has been suggested that co-deletion of several other contiguous genes (*OMG, RFN135, SUZ12* and *CENTA2*) or additional genetic aberrations in other

genes (*PTEN, TP53*) or non-coding RNA sequences should be responsible for the more severe phenotype, suggesting an interference of additional genetic factors (modifier genes) in modulating disease expression.[33-35]

Interesting, recent studies suggested a possible role of the lncRNA ANRIL and the chromosome 9p21.3 region as an important susceptibility locus for several specific tumoral hystotype in NF1.[36] ANRIL spans a 126.3 kb regions and it is transcribed into multiple tissue-specific splicing variants (SNPs), suggesting a physiological relevance and underlining the complexity of its regulatory function.[37,38]

ANRIL main function is to regulate its neighbour tumour suppressors gene CDKN2A/CDKN2B by transcriptional repression of the *CDKN2A-ARF-CDKN2B* locus in 9p21.3 with two different mechanisms: initiation of long term repression of CDKN2B locus by complexing SUZ12 in the polycomb repressive complex 2 (PRC2),[39] and maintenance of chromatin silencing of the CDKN2A/B locus through interaction with CBX7 in the polycomb repressive complex 1 (PRC1).[37]

Overall loss of function of the 9p21.3 locus was found in a wide variety of tumours, including plexiform neurofibromas, atypical neurofibromas and MPSNT, suggesting a potential role in genesis and progression of NF1-related tumours.[38,40]

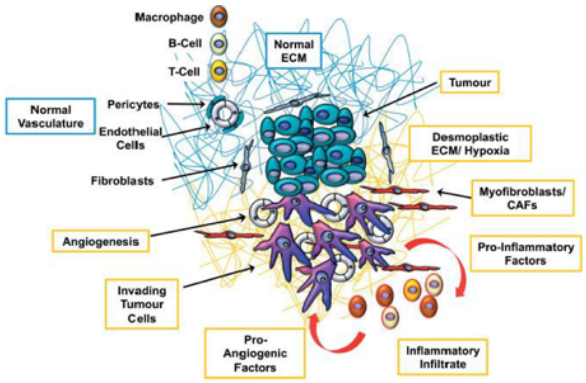
### *1.3.3 Tumour Microenvironment*

Tumour development and progression is not only determined by the corresponding tumour cells but also by the tumour microenvironment (TME).

The tumour microenvironment includes an orchestrated network of initially non-malignant interacting cell types, represented by immune cells, fibroblasts, pericytes, endothelial cells,

mesenchymal stroma/stem cells and sometimes adipocytes, which develop tumour-associated functionalities together with soluble factors (cytokines, chemokines, growth factors and various metabolites) and extracellular matrix (ECM) components, that all communicate with cancer cells thereby inhibiting and promoting tumorigenesis. TME can interact directly and indirectly with cancer cells by mutually altering properties and functions of the involved partners, establishing a certain immune status, contributing to blood vessel formation and neovascularization, and building an extracellular matrix which enables the associated cell populations to communicate within this microenvironment.[41]

It is important to strengthen that the TME is a dynamic and heterogeneous environment whose total composition varies between tumours and patients. However, the tumour stroma exhibits common features of these distinct cell types which may serve as interesting therapeutic targets, as after described for the plexiform neurofibroma.




*The tumour microenvironment*

## PLEXIFORM NEUROFIBROMA

Plexiform Neurofibromas (PNFs) are one of the primary features of Neurofibromatosis type 1 that occur in 30% (when defined clinically) to 56% (when identified by imaging) of NF1 patients.[1]

According to the WHO classification, PNF is a low grade tumour (grade I) defined by involvement of multiple fascicles, which are expanded by tumour cells and collagen, but commonly demonstrate residual, bundled nerve fibres at their centres; tumour consists of neoplastic well-differentiated Schwann cells intermixed with non-neoplastic elements including perineural-like cells, fibroblasts, mast cells, a variably myxoid to collagenous matrix and residual axons or ganglion cells.

These lesions typically present at birth but may continue to appear through late adolescence and early adulthood, having a significant size range, typically large and extensive, with irregular complex shapes. Their growth rate is erratic and unpredictable even if longitudinal studies observed that PNFs of younger individuals aged under 13 years grew faster than in older ones (2 $cm^2$/year and < 1$cm^2$/year respectively) exceeded the increase in their BMI.[56]

Tumours are classified by radiologist evaluation into three categories, based on complexity and morphological patterns:[42]

1. simple tumour: lesion with sharp boundaries, without extension or infiltration to adjacent tissue;
2. intermediate tumour: lesion with sharp boundaries, with extension or infiltration to adjacent tissue;

3. complex tumour: lesion with blurred margins, in which it was difficult to distinguish between tumour and surrounding tissues, with extension or infiltration to adjacent tissues.

PNFs are a significant cause of morbidity and mortality due to their propensity for local invasion, bone erosion, displacement and compression of surrounding structures, chronic pain and untoward aesthetics, in addition to their contribution to MPNST (malignant peripheral nerve sheath tumour) pathogenesis. [1]

Indeed, patients with plexiform neurofibromas have a lifetime risk of about 13-15% to develop MPNST, a high grade sarcoma that may include divergent differentiations with rhabdomyoblastic elements or glandular elements; little more than 20-25% of patients surviving the malignancy. Suspicious elements for malignant transformation are persistent or nocturnal pain, rapid growth, as well as the sudden onset of neurological deficits.[32]

Due to their nature, plexiform neurofibromas are highly refractory to radiotherapy and chemotherapy, and surgery is often extremely challenging because of localization of these lesions. In view of the limited viable treatment options, the lifetime risk of malignant transformation and the significant clinical consequences of PNFs, novel therapeutic options are needed.[32]

### *1.4.1 The Plexiform Neurofibromas Microenvironment*

As indicated above, dynamic interactions between tumorigenic cells and surrounding cells can dictate tumour initiation, progression and transformations. At the tissue level, plexiform neurofibromas demonstrate a complex microenvironment composed of Schwann cells, fibroblasts, perineural cells, mast cells, secreted collagen and blood vessels. At cellular

level, specific interactions between these cells engender tumour initiation and progression: tumorigenic Schwann cells secrete pathological concentration of stem cell factor (SCF), which recruit c-Kit expressing mast cell. In turn activated mast cells, release inflammatory effectors that stimulated the tumorigenic Schwann cells and their supporting fibroblasts and blood vessels, promoting tumour expansion in a feed-forward loop.[43]

Mast cells are granular, tissue-resident immune effector cells derived from early myeloid progenitor cells in hematopoietic tissue. Upon activation of the high-affinity IgE receptor (FcƐRI) and/or the c-kit receptor tyrosine kinase, mast cells release inflammatory mediators including histamine, serotonin, proteoglycans, and leukotrienes. In various disease models, mast cells can both positively and negatively regulate inflammation.[44]

In NF1-associated plexiform neurofibromas, SCF-recruited mast cells not only pervade tumour tissue and promote cellular growth, but they appear to be required for tumorigenesis; these insights have led to a promising therapeutic strategy.[46]

Mouse models have clarified the role of cellular and molecular interactions underpinning PNF formation: tumor development specifically requires the inflammatory contributions of $Nf1^{+/-}$ and c-kit dependent bone marrow and, arguably, hyperactive mast cells.

At first, Zhu et al. created a Schwann cell-specific *Nf1* conditional knockout on an *Nf1* haploinsufficient background, the first successful mouse model mimicking human neurofibroma genesis. This mouse carries Cre recombinase placed under control of the Krox20 promoter (Krox20cre), a promoter element preferentially expressed in a subset of Schwann cells. While mice with $Nf1^{-/-}$ Schwann cells but wild-type (WT) cellular backgrounds ($Nf1^{flox/flox}$; Krox20cre) never develop neurofibromas, mice with additionally heterozygous backgrounds ($Nf1^{flox/-}$; Krox20cre) reliably form dorsal root ganglia tumors

comprised of irregular Schwann cells, fibroblasts, and mast cells. These findings grossly and histologically mimic plexiform neurofibromas found in individuals with NF1.[46]

This study suggested that plexiform neurofibroma development requires $Nf1^{-/-}$ Schwann cells, analogous to *NF1* loss of heterozygosity (LOH) in humans, but also an *Nf1* haploinsufficiency ($Nf1^{+/-}$) of cellular components within the tumor microenvironment cellular background.[46]

A successive study of Yang et al. on bone marrow transplantation experiments in this mouse model demonstrated that neurofibroma formation hinges on *Nf1* haploinsufficient and c-kit-dependent bone marrow. First, $Nf1^{+/-}$ marrow transplanted into lethally irradiated $Nf1^{flox/flox}$; Krox20cre mice induces tumor formation similar to the tumorigenic $Nf1^{flox/-}$; Krox20cre mouse model. Second, transplantation of WT bone marrow into the $Nf1^{flox/-}$; Krox20cre mouse abolishes tumor formation in the normally reliable tumor model. In the first experiment, the mouse carries $Nf1^{-/-}$ Schwann cells, $Nf1^{+/-}$ marrow, and WT fibroblasts, vascular cells, neurons; in the second experiment, the mouse carries $Nf1^{-/-}$ Schwann cells, WT marrow, and $Nf1^{+/-}$ fibroblasts, vascular cells, neurons. Therefore, the authors concluded that $Nf1^{+/-}$ bone marrow, combined with $Nf1^{-/-}$ Schwann cells, is required and sufficient for plexiform neurofibroma formation.[43,45]

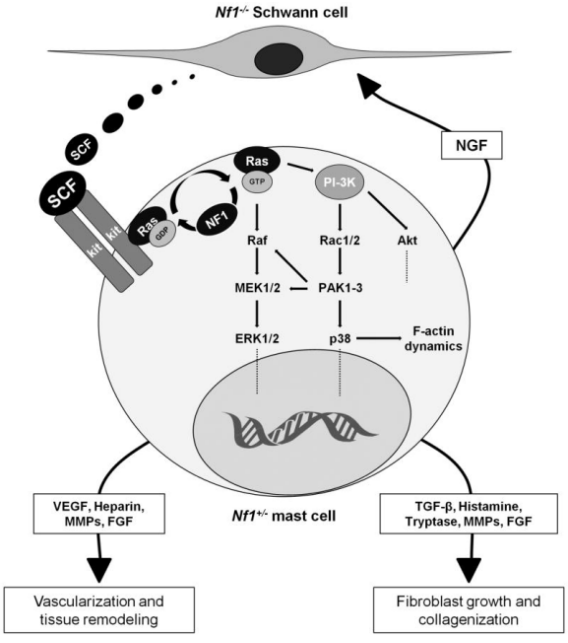
Furthermore, the same authors crossed $Nf1^{+/-}$ mice with mice carrying distinct mutations (*W*), which compromise c-kit receptor tyrosine kinase, the principal molecular effector of mast cell development. Marrow derived from $Nf1^{+/-}$ mice intercrossed with $W^{41}$ or $W^{sh}$ mice and transplanted into $Nf1^{flox/flox}$; Krox20cre mice does not induce tumor formation as in $Nf1^{flox/flox}$; Krox20cre mice reconstituted with $Nf1^{+/-}$ marrow.

Additionally, conclusion was that PNF formation in a mouse model requires Schwann cell *Nf1* deficiency, marrow *Nf1* haploinsufficiency, and marrow expressing high functioning c-kit receptors.[43-45]

The next question was which haploinsufficient cells and/or cellular interactions underlie neurofibroma genesis. The $Nf1^{+/-}$ mast cell seems a likely candidate: mast cells infiltrate neurofibroma tissue in large numbers, neurofibroma tissue express high levels of stem cell factor (SCF) messenger RNA, SCF/c-kit signaling critically regulates mast cell hematopoiesis and physiology mast cells and other inflammatory cells have been increasingly implicated in neoplasia. Likewise, mast cells can synthesize and secrete factors modulate the differentiation and growth of multiple cell types, effect extracellular matrix remodeling, and induce collagen deposition.[44]

In the current hypothesis, $Nf1^{-/-}$ Schwann cells co-opt and activate $Nf1^{+/-}$ mast cells through secreted high levels of SCF, driving the mast cells differentiation, proliferation, degranulation, and secretion cytokines. These activities, in turn, induce continued Schwann cell expansion, aberrant fibroblast bioactivity, the in-growth of new vasculature, and the perpetuation of marrow-based inflammation. As evidence, $Nf1^{-/-}$ Schwann cells release pathological concentrations of SCF under normal culture conditions, and this SCF provides a chemotactic and activating signal most potently for $Nf1^{+/-}$ mast cells. $Nf1^{-/-}$ Schwann cell-conditioned media drives $Nf1^{+/-}$ mast cell chemotaxis at approximately twice the rate of WT mast cells, an effect duplicated with recombinant SCF and ablated with genetic and pharmacologic disruption of c-kit. In turn, activated $Nf1^{+/-}$ mast cells secrete high levels of TGF-β, inducing $Nf1^{+/-}$ and WT fibroblasts to migrate, proliferate, and synthesize collagen, a protein comprising nearly half the dry weight of the tumour. Likewise, mast cells

potentiate Schwann cell-axonal disassociation in peripheral nerve injury and neuronal tumor models. SCF-activated mast cells produce a host of inflammatory and mitogenic factors with potential yet relatively unexplored roles in the plexiform neurofibroma microenvironment.[43,47] Biochemically, *Nf1*$^{-}$ and SCF-dependent mast cell pathophysiology results from deregulated Ras signalling. *Nf1*$^{-/-}$ Schwann cell secretes pathological concentrations of SCF, the ligand for the c-kit receptor tyrosine kinase on the *Nf1*$^{+/-}$ mast cell. C-kit dimerization activates Ras-Raf-MEK-ERK and PI-3K-Rac-Pak-p38 signalling pathways, which promote mast cell proliferation, survival, migration, and cytokine synthesis/secretion. These pathways crosstalk through Pak phosphorylation of Raf and MEK and are negatively regulated through NF1-GAP activity. Secreted products such as VEGF, TGF-β, NGF, and MMPs promote tumour vascularization, collagen deposition, Schwann cell expansion, and extracellular matrix remodelling, respectively. These products initiate and promote tumour growth.[43]




*Role of mast-cells in plexiform neurofibromas development*

### *1.4.2 A proposal of treatment: c-Kit inhibition*

Considering the data reported above implicating the *Nf1*$^{+/-}$ mast cells in tumor formation and their dependence on hyperactive c-kit pathways, pharmacological c-kit inhibition are supposed to modulate disease course. Concordantly, Imatinib Mesylate (Glivec/Gleevec©), a potent inhibitor of c-kit, platet-derived growth factor (PDGF), and the bcr/abl receptor tyrosine kinase, successfully reduces existing PNFs volume and *de novo* genesis in the *Nf1*$^{flox/-}$;Krox20cre mouse model.[45]

From the results of drug treatment in the mouse model, the same authors have used Imatinib in a compassionate use protocol to treat a three years old patient with NF1 and a progressive, not surgically resectable, and debilitating plexiform neurofibroma encasing her carotid artery and jugular vein. After three months of treatment with 350 mg/m$^2$/dose of Imatinib Mesylate, the tumor decreased in volume by 70%. Sleeplessness, fatigue, and drooling associated with airway compression resolved, and the patient remains stable and relatively healthy without further Imatinib treatment.[45]

On the basis of these findings, Robertson and colleagues started an open-label phase II trial to test whether treatment with Imatinib Mesylate can decrease the volume burden of clinically significant PNFs in patients with NF1.[48]

In this study, 36 NF1 patients aged 3-52 years with PNFs were enrolled and treated with daily oral Imtinib Mesylate at 220 mg/m2 twice a day in children under 19 years old, and 400 mg twice a day in adults; 23 of them, with a total of 69 tumours volumetrically measurable, completed the study drug receiving the treatment for at least 6 months while 13 patients withdrew from the study prematurely.

The authors reported an objective response to Imatinib Mesylate in 26% of the evaluable patients enrolled in the study, noting a profound response in a subset of PNFs, some of which reduced in volume by almost 40% with a median reduction of 26·5%. In this study, 36 NF1 patients aged 3-52 years with PNFs were enrolled and treated with daily oral Imtinib Mesylate at 220 mg/m2 twice a day in children under 19 years old, and 400 mg twice a day in adults. 23 of them, with a total of 69 tumours volumetrically measurable, completed the study drug receiving the treatment for at least 6 months while 13 patients withdrew from the study prematurely

The authors reported an objective response to Imatinib Mesylate in six of the evaluable patients (26%) enrolled in the study, noting a profound response in a subset of PNFs, some of which reduced in volume by almost 40% with a median reduction of 26,5%. Although eight (12%) of 69 individual tumours exceeded the 20% volume reduction threshold constituting treatment response, only one patient continued on long-term treatment. A further 36 (52%) tumours remained stable (<20% increase or decrease in volume) and 25 (36%) progressed (≥20% increase in volume).

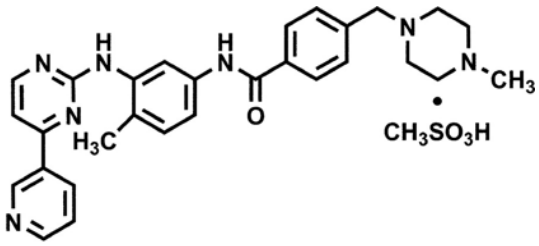
It's important to notice that a substantial subjective improvement of symptoms was reported by patients, including in someone in whom PNFs were stable.

The authors noted heterogeneity in response not only between patients, but also between different tumours in individual patients which probably results from the biology of PNFs partly be due to cells expressing the c-kit receptor in the tumour microenvironment as characterized in the pre-clinical model.

No statistical significant differences analysing the data respect to age, lesion location, and size were found. However larger PNFs tended to be less responsive and head and neck

plexiform neurofibromas seemed to be more responsive than the lesions localized in other body parts across age groups.[48]

### *1.4.3 Imatinib Mesylate*




Imatinib Mesylate (IM) is the first tyrosine kinase inhibitor and the paradigm of targeting molecular pathogenetic events in cancer. Developed as a specific inhibitor of the Bcr-Abl protein tyrosine kinase of chronic myelogenous leukemia (CML), the expanding understanding of the basis of imatinib-mediated tyrosine kinase inhibition has revealed a spectrum of potential other antitumor applications beyond the powerful activity already reported in the treatment of CML.[49]

The primary action of IM is the selective competition with ATP in a specific binding site of the Bcr-Abl tyrosine kinase fusion protein, constitutive expressed in CML due to the formation of the *bcr-abl* oncogene.

In addition to various oncogenic forms of the Bcr-Abl, imatinib selectively inhibits the ABL-related protein (ARG), the platelet-derived growth factor (PDGF) receptor and Kit.

PDGF is a connective tissue cell mitogen that has also been shown to play an important role in tumorigenesis. The active PDGF dimeric isoforms bind to two structurally similar tyrosine kinase receptors, PDGF-alfa and PDGF-β activating a number of intracellular signalling pathways that ultimately promote cell growth, changes in cell morphology and

prevention of apoptosis. IM compete selective with PDGF receptors showing activity in vivo against PDGF-driven tumours models including gioblastoma, dermatofibrosarcoma protuberans and chronic myelomonocytic leukemia.[49]

Furthermore, PDGF- β receptor is expressed on vascular endothelial cells and it has been shown to have angiogenic activity in various models, acting not only as a direct mitogen for endothelial cells, but also inducing expression of vascular endothelial growth factors (VEGF) which in turn causes an autocrine loop through stimulation of VEGF receptors. Since PDGF-responsive stromal and perivascular cells are a major source of VEGF, PDGF may support indirectly blood vessels formation and influenced angiogenesis through recruitment of pericytis and stimulation of vascular smooth muscle cells. Based on these observation, imatinib was investigates for possible antiangiogenic activity, confirming antiangiogenetic effects in inhibition of PDGF, VEGF and bFGF-induced angiogenesis in vivo, resulting in inhibition of tumour angiogenesis, growth and progression.[49]

IM is also a potent inhibitor of the Kit receptor tyrosine kinase and it has demonstrated clinically activity against the Kit-driven tumours. The *c-kit* gene product is expressed in hematopoietic progenitor cells, mast cells and germ cells and mutations of *c-kit* resulting in SCF ligand-indipendent activation of the receptor have been identified in a number of tumour types by Ras-pathway stimulation.[49]

Therefore, in NF1-associated plexiform neurofibromas, Imatinib Mesylate acts inhibiting the SCF/c-kit signaling that regulates mast cell, critically required elements involved in tumorigenesis. The drug may also contribute through simultaneous inhibition of PDGFR and c-abl, signaling molecules involved in *Nf1*-dependent angiogenesis and fibroblast activity, respectively.[48,49]

### *1.4.4 Biological Markers*

Medical progress requires the discovering and clinical use of biomarkers useful for early detection of disease or in disease classification (diagnostic biomarkers), in predicting response or adverse events (predictive biomarkers), in defining optimal drug dose (metabolic/pharmacodynamic biomarkers) or in forecasting progression or recurrence (outcome biomarkers).[50]

For Neurofibromatosis type 1, all biomarkers published to date are considered exploratory and none have been validated. Furthermore, most of the NF1 research focused on complex molecular predictive biomarkers and candidate gene alterations.[50]

The opportunity to study novel agents in the disease opens the necessity of developing and incorporating pharmacodynamic and predictive serum biomarker studies into early clinical trials.

In relation to the Imatinib Mesylate action mechanism and the importance of angiogenesis in tumours pathogenesis and progression, circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) are promising candidate to serve as such markers. Indeed, preclinical and clinical data suggest that CECs might be useful to identify patients who might benefit from anti-angiogenetic treatments, while EPCs seem to have a "catalytic" role in different steps of cancer progression and recurrence after therapy.

CECs are mature endothelial cells released from vessels during endothelial turnover and EPCs are a subpopulation of CECs that are mobilized from the bone marrow to complement local angiogenesis. [51]

In specific types of cancer (including some central nervous system tumours), CECs number

and viability are increased when compared to healthy control. This is probably due to the angiogenic switch associated with cancer growth and the related production of angiogenic growth factor and count of circulating endothelial cells can be used as a surrogate biomarker for angiogenesis and anti-angiogenetic drug activities. Furthermore, an increased in the number of apoptotic CECs is associated with prolonged free-survival in several diseases.[51]

Increased in ECPs numbers is correlated to a worse prognosis in cancer patients; indeed, these cells appear to have a transient critical role in promoting angiogenesis during tumour growth, in stimulating metastasis and in in rebound revascularization after certain therapies are stopped. These cells are potentially targets for anticancer therapies and for adjuvant therapeutic strategies in patients at risk for cancer relapse.[51]

## AIM OF THE STUDY

Plexiform Neurofibromas are invasive and growing tumours, with substantial clinical consequences including severe disfigurements, pain, organ compression and other functional impairments related to infiltration, displacement and compression of the surrounding structures. Furthermore, PNFs can result in substantial morbidity and early mortality due to a potential risk of transformation into malignant peripheral nerve sheath tumours (MPNSTs); no effective therapeutic options are really available.

Primary aim of this study is to test the hypothesis that inhibition of c-kit signalling pathways by Imatinib Mesylate results in objective reduction and/or inhibition of the growth of progressive, not surgically resectable plexiform neurofibromas (PNFs) in NF1 patients, according to the recent data reported in literature.

Furthermore, potentially predictive and prognostic biological markers of tumour progression and response to treatment were investigated. Considering the mechanism of action of Imatinib Mesylate and the proven role of microenvironment in tumour genesis and evolution, we investigated the possible value of angiogenic factors (circulating endothelial cells and progenitors) as targets and response indicators for the tyrosine kinase inhibitor treatment.

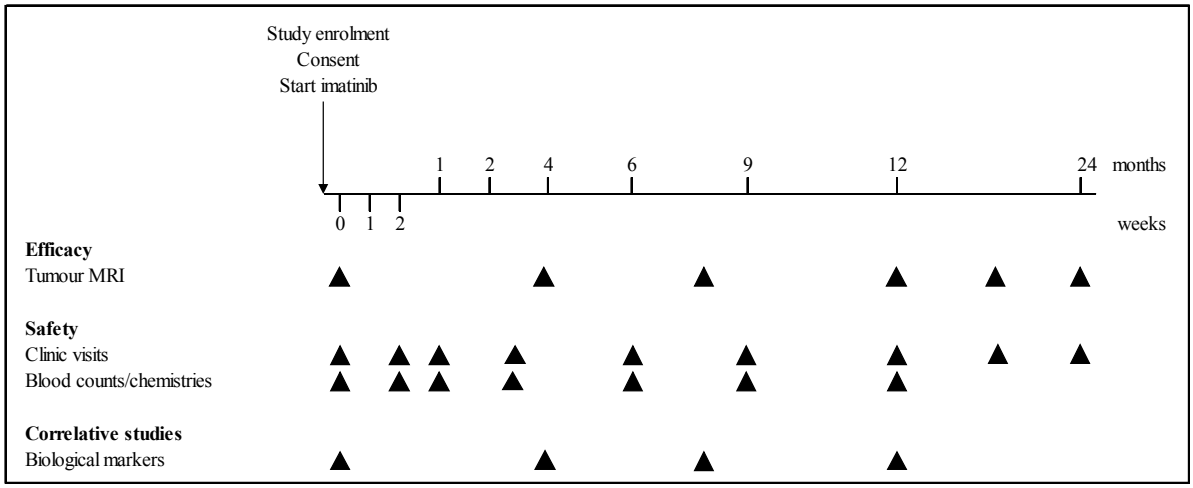
## STUDY DESIGN

This study was approved by the Italian Ministry of Health and it allowed the inclusion of only 6 NF1 patients. Written informed consent was obtained from all subjects.

It was designed as follow:

- as proposed by Robertson and collegues,[48] orally administration of Imanitib Mesylate at the dose of 220 mg/m$^2$ twice a day for children and 400 mg twice a day for adults, for 12 months unless severe toxicity;
- Pre-treatment evaluation including history and clinical examinations and assessment of quality of life scales appropriated to age, measurement of baseline PNFs size by standardized MRI protocol, blood counts and comprehensive blood biochemistry, urine analysis and B-HCG dosage for woman of childbearing age, dosage of circulating endothelial cells (CEC) and progenitors (CEP) serum levels;
- Histopathological confirmation diagnosis according to WHO criteria and assessment of c-KIT expression on PNFs biopsy or partial resected;
- During the 12 months of the drug administration, a follow-up visit including review of symptoms and quality of life assessment, physical examination, complete blood count and serum chemistries, every 3 months or as clinically indicated;
- Graduation of adverse events according to the National Cancer Institute Common Terminology Criteria (version 4.03),[52] and Imatinib Mesylate dose adjustments for grade 3 and 4 adverse effects;
- Follow-up MRI studies, and CEC and CEP serum levels every 4 months during the treatment;

- After the drug discontinuation, clinical and neuroradiological evaluations every six months or as clinically indicated for the following 12 months.




*Study design*

## METHODS and PROCEDURES

### *4.1 Patient eligibility*

Participants were drawn from a cohort of 350 patients with a clinically and/or genetically confirmed diagnosis of Neurofibromatosis type 1 (NF1), admitted to the Carlo Besta Neurological Institute, a tertiary care referral centre. All patients were enrolled in a longitudinal follow-up study, with the same clinical guidelines.

Inclusion criteria were the following:

- clinical diagnosis of NF1 according to the National Institutes of Health (NIH) criteria;
- presence of a disabling or deforming plexiform neurofibroma (PNFs), defined as a tumour causing a compression to the spinal cord or impinging on vital organs, or tumours that significantly impair patients' quality of life from a subjective point of view, for example due to pain or other symptoms depending on the tumours' location, or a major cosmetic impairment;
- Karnofsky[53] or Lansky[54] performance score level of 50 or more;
- c-kit expression on tumour biopsies.

Exclusion criteria were the following:

- chemotherapy exposure or any other investigational agents in the 28 days before enrollment to the present study;
- presence of a growing tumour requiring radiation or chemotherapy;
- history of other malignancies during 5 years previous to enrollment to the present study;
- uncontrolled medical disease and presence of class III or IV (according to New York Heart Association criteria) heart failure;

- pregnancy or breastfeeding;
- presence of HIV infection;
- previous radiation to 25% or more of bone marrow space;
- history of a major surgery within 2 weeks before enrollment to the present study;
- a significant concern for medical non-compliance.

6 patients (3 females), aged 3–65 years, were enrolled in the present study. A further child was included in the study within a compassionate use program.

### 4.2 *Clinical Evaluation and Quality of life assessment*

A detailed clinical and neurological examination, and a medical history focused on the presence of symptoms dependent to PNFs location, on previous surgical or pharmacological treatments and on the functional status of the patients according to Karnofsky or Lansky performance scale, were performed for every patient.

The Karnofsky scale is designed for subjects aged 16 years and older, while the Lansky scale for patients less than 16 years old; both the scales are based on general well-being and activities of daily life and score ranges between 0 (dead, unresponsive) and 100 (normal, fully active).[53,54]

Health-related quality of life (HRQOL) for investigate the subjective perceived health based on physical functioning, mental health, cognition and emotion, was analysed using WHO-DAS scale (World Health Organization Disability Assessment Schedule) a 36-item version self-administered questionnaire about difficulties due to health condition. It' s a generic assessment instrument that make a standardized levels and profiles of the disability

and that covers six domains of functioning including cognition, mobility, self-care, life activities, participation in community activities and interaction with other people.[55]

Furthermore, in children a comprehensive protocol applying the ICF version for Children and Youth (International Classification of Functioning, Disability and Health, Children and Youth version), the quality of life KIDSCREEN questionnaires and the Vineland assessment was performed at three time-point.[56]

### *4.3 Routine Laboratory test*

For every patient urine analysis, complete blood count and serum chemistries including total bilirubin, aspartate and alanine aminotransferase, alkaline phosphatase, creatinine, azotemia, were performed before starting treatment and then weekly for 2 weeks, monthly for 1 month and regularly every 3 months thereafter or as clinically indicated.

### *4.4 Magnetic Resonance Imaging*

All imaging was done on a 1.5 T clinical MRI (Magnetom Avanto, Siemens Medical Solutions, Malvern, PA, USA) using coronal and axial short T1 inversion recovery (STIR) images at 5-10 mm gapless slice thickness to encompass the entire PNF. STIR sequence, which suppresses fat signal and accentuates the water signal, is able to easily differentiate neurofibromas from surrounding tissue without the addition of intravenous gadolinium. Subsequently, volumetric sequences were performed. Each wide PNFs were measured on sequential MRI sections by manually outlining the tumour, and with an automated method the total tumour volume was obtain. Due to the wide extension of PNFs, the volume was expressed in millilitres (mL) and the main tumour location was defined as the body area where >80% of the lesion was localized.

Response to the treatment was defined as a sustained 20% or more reduction in tumour volume from baseline in two or more sequential MRIs, including the MRI at the conclusion of the study protocol. Progression was defined as a 20% or more increase in lesions volume; PNFs that showed less than 20% reduction and less than 20% increase in volume were categorized as stable.

### *4.5 Immunohistochemistry (IHC)*

Patients' specimens were fixed in Carnoy's solution, while the correspondent xenografted mouse brains were formalin-fixed and then both dehydrated, paraffin-embedded and sectioned at 2μm according to established procedures. For hematoxylin-eosin staining, slides were stained in Carazzi hematoxylin solution, rinsed in running tap water and counterstained in eosin solution. Antigen's retrieval was performed at pH 9 and 90°C in a PT Link pre-treatment module (Dako, Glostrup, Denmark) when requested. Slides were first blocked in 3% H2O2 (Sigma-Aldrich, St. Louise, Missouri, USA), then they were incubated with Normal Goat Serum (Dako, Glostrup, Denmark) and with the respective primary antibodies: rabbit polyclonal anti-cKIT (1:100, Dako, Glostrup, Denmark), rabbit polyclonal anti-PDGFRβ (1:200, Santa Cruz Biotechnology, USA). Sections were subsequently incubated with anti-rabbit Envision® peroxidase conjugated (Dako, Glostrup, Denmark) as secondary antibody, for 1 hour at room temperature. Finally, slides reacted with diaminobenzidine (DAB Substrate Chromogen System, Dako Cytomation, Glostrup, Denmark), counterstained with hematoxylin, mounted and visualized using a bright field microscope.

### *4.6 Biological Markers*

In relation to the Imatinib Mesylate action mechanism and the proven role of angiogenesis in tumours pathogenesis and progression, circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) were evaluated as target and response indicator for the tyrosine kinase inhibitor treatment.

Number and viability of CECs and CEPs were measured on fresh samples from the 7 patients at baseline and every 4 months during the treatment by six-color flow cytometry. Blood samples were collected in EDTA discarding the first 3 ml of blood to avoid contamination with endothelial cells from venipuncture. The samples were kept at room temperature (22±2 °C) and processed as within 24 hours after collection.

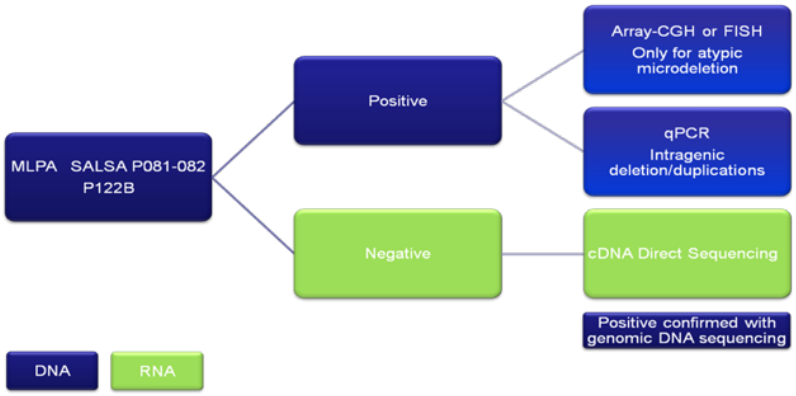
CECs were defined as Syto16(DNA)+CD31+CD109 and EPCs as Syto16(DNA)+CD45-CD34+; percentage of apoptosis was evaluated.

We also investigated the levels of Syto16(DNA)+CD45dimCD34+VEGFR2+, described as VEGFR2+ hematopoietic progenitor cells, and Syto16(DNA)+CD31-CD140b +PPC as regulator of vessel stability and vascular survival.[51]

To define reference value, cells subpopulations were investigated in healthy controls matched for age and sex.

### *4.7 Molecular study*

Molecular study of *NF1* gene was performed in every patient using a multistep method comprising both DNA and RNA approaches.




*Multistep molecular study comprising both DNA and RNA approaches*

*DNA extraction, RNA Extraction and Retro-transcription:* DNA was isolated from EDTA-blood samples using Gentra ®Puregene® Blood Core Kit B (Quiagen, Venlo, Netherlands). RNA samples were collected in Tempus Blood RNA Tube (Life Technologies) and extracted with Tempus$^{TM}$ Spin RNA Isolation Kit within 4 days. DNase treatment with Absolute RNA Wash Solution was performed for all samples during RNA extraction protocol. RNA samples (1ug) were reverse-transcribed using 50 units of High-Capacity cDNA Reverse Transcription kit (Life Technologies) and 20 units of RNAse inhibitor (Ambion®). Beta2microglobulin amplification was used as quality control for retro-transcription.

*Multiplex Ligation-Dependent Probe Amplification Analysis (MLPA) :* Patients' DNA was analyzed by MLPA with NF1 MLPA salsa P081 and P082 (MRC Holland, Amsterdam, The Netherlands). P081/P082 salsa kit highlighted single- and multi-exon deletion/duplications inside the NF1 gene. The P081 probemix-1 contains 38 MLPA probes and the P082 NF1 promix-2 41 MLPA probes. The amplification products are between 130 and 463 nt, covering almost all 60 exons of the *NF1* gene. P081/P082 positive patients for

the entire NF1 deletion were screened also with MLPA P122 salsa kit to determine large duplications/deletions or microdeletions. This kit revealed microdeletions that involved NF1's contiguous genes thanks to the presence of 25 probes for 17 different genes closely located to NF1. Results obtained by ABI Prism 3130 Genetic Analyzer (Life Tecnologies) were analyzed with the Coffaliser.Net Software (MRC Holland, Amsterdam, The Netherlands).

*PCR amplification and cDNA Sequencing: NF1* cDNA were fully amplified with 22 overlapping PCR using Taq Gold Polymerase® (Applied Biosystems, Foster City, CA). The fragments were designed (about 400 bp) in according to Valero et al. 2011 but with a new primer pairs for fragments from exon 20 to 23.2 and from exon 29 to exon 31. PCR products were purified using ExoSAP-IT® (USB Corporation USA) according to the manufacturer's protocol and sequenced in both directions using the ABI BigDye terminator sequencing kit v3.1 (Life Technologies) on 3500xL Genetic Analyzer (Life Tecnologies). cDNA variations were confirmed at DNA level and Exon 1 and 23a were usually sequenced also on DNA to test GC rich regions. cDNA mutations were confirmed at DNA level.

### *4.8 Data and Statistical analysis*

A qualitative data analysis was performed to evaluate longitudinal change of clinical (including healthy related quality of life), biological and neuroradiological data from baseline to the follow-up.

The non-parametric Spearman correlation test was performed to investigate the relationship between the clinical variables and severity score. To study the trend of the clinical variables

scores at baseline, time 1 after 4 months and time 2 after 8 months, the nonparametric Friedman test for one-way repeated measures analysis of variance by ranks was used. The Wilcoxon signed-rank test, a nonparametric test of non-independent data from only two groups, was performed to understand if the difference was significant between baseline and time1, time1 and time2, or baseline and time 2 measures.

Finally, the non-parametric Mann-Whitney test for two independent groups was used to compare the patients with healthy controls matched for sex and age.

One-tailed exact p values of 0.05 or lower were considered significant.

# RESULTS

## *5.1 Patients characteristics*

Participants were drawn from a cohort of patients with a clinically and/or genetically confirmed diagnosis of Neurofibromatosis type 1 (NF1), admitted to the Carlo Besta Neurological Institute, a tertiary care referral centre. A total of 7 individuals fulfilled the eligibility criteria and were therefore enrolled in the present study.

***Patient 1*:** BS, a 5-year-old female with familial NF1 genetically confirmed (c.1721+3 A>G), was followed since birth for a hypertrophy of the clitoris at another tertiary care referral Centre. The work-up evaluations revealed the presence of extensive confluent plexiform neurofibromas extended from retroperitoneal region (periuterine, perivescical and rectal levels) to external genitalia, bilateral gluteal regions and posterior compartment of lower limbs through the sciatics foramina. The PNFs were in slow but continuous numerical and volumetric progression as confirmed to the periodic subsequent clinical and imaging (MR and ultrasound) controls. No surgical intervention was proposed due to the localization and extension of the tumour.

Pre-treatment physical examination revealed the presence of café-au-lait macules on the trunk and arms, freckling in the axillary region, hyperpigmented and hypertrophic soft area in the right arm, and a wide hyperpigmented and hypertrichotic area extend to genital, dorsal, gluteal and posterior lower limbs levels. Hypertrophy of the clitoris and external genitalia was also present.

Blood counts and comprehensive blood biochemistry and urine analysis confirmed an adequate end-organ function. Basal serum levels of circulating endothelial cells were analysed. Baseline MRI documented a total volume of PNFs of about 3784 ml.

Treatment with Imatinib Mesylate was administrated orally at the dose of 220 mg/m$^2$ twice a day (Glivec 100 mg + 200 mg) for 12 months. No toxicity and adverse event were recorded throughout the whole period of drug administration.

Over the drug administration period, the growth of PNFs was well documented using the MRI method and the tumour volume increased of 3.7% per year.

The evaluations of the general intellectual ability revealed age-appropriate cognitive skills (WPPSI-III, Q.I.T.= 100), with homogeneous profile into all the cognitive domain investigate. Cognitive abilities and adaptive-social functioning remained age-appropriate during different clinical follow-up, as well as the quality of life perceived consistently good and no influenced by the general clinical condition and the pharmacological treatment.

12 months after discontinuation of treatment, clinical and neuroradiological stabilities was recorded (growing rate 2.5% per year).

***Patient 2:*** MN, a 12-year-old male with familial NF1 genetically confirmed (a 13 kb multiple exons deletion), was followed since three months of life for diffuse café-au-lait spots at another medical Centre. During the follow-up, at 10 years of age progressive chest and left paravertebral/gluteal painful masses were noted and MRI confirmed the presence of a wide retroperitoneal PNFs with sigmal, vescical and right kidney compression and extension to to right gluteal region, in addition to multiple intra and extradural spinal neurofibroma compressing the spinal cord at C3-C5 level.

During the first evaluation at our Institute, due to the grade of the cervical spinal cord compression and the presence of neurological signs, surgical excision of C3-C4 neurofibromas was needed with consequent right hemiparesis and a left Bernard-Horner syndrome. Afterwards treatment with Imatinib Mesylate was proposed.

Pre-treatment physical examination revealed the presence of diffuse café-au-lait macules, inguinal and axillary freckling, multiple subcutaneous neurofibromas, soft masses at left pectoralis, right gluteal and omolateral lumbar levels. Partial resection of the gluteal components was performed.

An adequate end-organ function was confirmed by complete blood and urine analysis. Basal serum levels of circulating endothelial cells were measured. Baseline MRI documented a total volume of PNFs of about 4541 ml.

Treatment with Imatinib Mesylate was administrated orally at the dose of 220 mg/m$^2$ twice a day (Glivec 200 mg + 200 mg) for 12 months. No toxicity and adverse event were recorded throughout the whole period of drug administration.

Over the drug administration period, the volume of PNFs increased of 3% per year.

The evaluations of the general intellectual ability revealed age-appropriate cognitive skills (WISC-IV: Q.I.T. = 110), with a profile characterized by worse performance in verbal comprehension, processing speed and working memory. Cognitive abilities remained age-appropriate during the follow-up. The administration of quality of life scale revealed a fluctuating depressive state and adaptive-social functioning, principally due to the dismissed hopes of a greater improvement following the initiation of therapy.

12 months after discontinuation of treatment, clinical and neuroradiological stabilities was documented (growing rate 4% per year).

***Patient 3:*** SM, a 11-year-old female with familial NF1 genetically confirmed (c.1527+675 C>T) was followed since 21 months of life for a hypertrophy of the clitoris at another tertiary care referral Centre. The instrumental evaluations revealed the presence of an optic pathway glioma, multiple spinal neurofibromas, pelvic and abdominal plexiform neurofibromas and concomitant histologically assessed presacral ganglioneuroma. Despite of multiple treatments, including chemotherapy, radiotherapy and antiangiogenic drug, the PNFs were in slow but continuous numerical and volumetric progression, and a bilateral percutaneous nephrostomy was needed due to kidney and urinary tracts compression and consequent severe hydronephrosis. No surgical intervention was proposed due to the localization and extension of the tumour. Compassionate use of Imatinib Mesylate was proposed.

At the first clinical examination at our Institute, café-au-lait macules, axillary and inguinal freckling, subcutaneous neurofibromas were recorded. A wide painful and deforming left gluteal and abdominal mass was present.

Blood counts and comprehensive blood biochemistry, and urine analysis confirmed value referable to chronic renal insufficiency; basal serum levels of circulating endothelial cells were analysed. Baseline MRI documented a total volume of PNFs of about 6683 ml.

Treatment with Imatinib Mesylate was administrated orally at the dose of 220 mg/m$^2$ twice a day (Glivec 200 mg + 200 mg) only for 2 months; for the following 10 months, the treatment was administred at a lower dosage (Glivec 200 mg) associated with Furosemide, due to worsening of renal function (grade 3 AE), diffuse oedema (grade 3 AE) and weight gain (grade 3 AE).

Over the drug administration period, the PNFs volume increased of 2.9% per year.

The evaluations of the general intellectual ability revealed age-appropriate cognitive skills (WISC-III: Q.I.T. = 114), with a homogenous profile. Cognitive abilities and adaptive-social functioning remained age-appropriate during different clinical follow-up; quality of life was perceived consistently good and no influenced by the pharmacological treatment. With regards to the behavioral-emotional aspects, difficulties consequent to the management of the clinical situation were referred, against high levels of tolerance and adaptability of the children.

During the treatment, the progression of a thoracic atypical neurofibromas was documented diagnosed as MPNST 10 months after drug discontinuation; the patient died 6 months later. Regarding PNFs evolution, a growing rate of 4.8% per year was recorded after interruption of IM dosing.

***Patient 4:*** MF, a 18-years-old male with sporadic NF1 genetically confirmed (c.5493 G>T), was followed since the age of two for café-au-lait macules and a right hand plexiform neurofibroma. During the follow-up, progressive paravertebral PNFs were documented on MRI, in addition to multiple intra and extradural spinal neurofibromas compressing the spinal cord at C2-C5 level. Due to the grade of the cervical spinal cord compression and the presence of neurological signs, surgical excision of cervical neurofibromas was needed. Afterwards treatment with Imatinib Mesylate was proposed in relation to PNFs progression.

Pre-treatment physical examination revealed the presence of diffuse café-au-lait macules, inguinal and axillary freckling, multiple soft masses referable to plexiform neurofibromas (right hand, left leg and at chest level).

Blood counts and comprehensive blood biochemistry, and urine analysis confirmed an adequate end-organ function; basal serum levels of circulating endothelial cells were analysed. Baseline MRI documented a total volume of PNFs of about 3873 ml.

Treatment with Imatinib Mesylate was administrated orally at the dose of 300 mg twice a day for 3 months, then reduced at 200 mg twice a day due to diffuse paraesthesia (grade 2 AE). After 5 months of overall treatment, the drug was discontinued due to neurological worsening and progression of grade of the cervical spinal cord compression with need of another surgical intervention.

The growing rate of the plexiform neurofibromas volume was of 2.4% (2.7% per year, after drug discontinuation).

The evaluations of the general intellectual ability revealed cognitive abilities remained age-appropriate during the follow-up. The administration of quality of life scale revealed a fluctuating depressive state and adaptive-social functioning related to the clinical worsening and the necessity of repeated surgical treatments.

***Patient 5:*** CD, a 19-years-old female with sporadic NF1 genetically confirmed (c.1721+3 A>G), was followed since the first years of life for diffuse café-au-lait spots, optic pathway glioma and freckling at another medical Centre. During the follow-up, at 18 years of age she presented nocturnal pain at left elbow and a MPNST was diagnosed and surgically removed after 4 cycles of chemotherapy. Subsequent other 4 cycles of chemotherapy were performed.

Furthermore, a progressive sacral and right gluteal painful mass was noted and MRI confirmed the presence of a wide sacral and parvertebral PNFs, histhopathologically confirmed, and the treatment with Imatinib Mesylate was proposed.

Pre-treatment physical examination revealed the presence of diffuse café-au-lait macules, inguinal and axillary freckling, multiple subcutaneous neurofibromas and right gluteal mass.

An adequate end-organ function was confirmed on blood and urine analysis, and the basal serum levels of circulating endothelial cells were evaluated. Baseline MRI documented a total volume of PNFs of about 2273 ml.

Imatinib Mesylate was administrated orally until the dose of 500 mg a day for 4 months and then the treatment was discontinued for malignant progression of a left axillary atypical neurofibroma into MPSNT. During the treatment the patients lamented severe diffuse paraesthesia (grade 3 AE) and the growing rate of PNFs volume was of 1.8%.

Cognitive abilities and adaptive-social functioning remained age-appropriate during different clinical follow-up; during the study drug, a slight improvement in quality of life scales was observed.

12 months after discontinuation of treatment, PNFs stability was documented (growing rate 3.9% per year).

***Patient 6:*** GC, a 35-years-old male with familial NF1 genetically confirmed (c.6801 A>G), was followed since the age of eight for diffuse café-au-lait spots, axillary and inguinal freckling, multiple spinal neurofibromas and progressive paraspinal, pelvic and abdominal PNFs surgically treated several times.

Pre-treatment physical examination revealed the presence of diffuse café-au-lait macules, inguinal and axillary freckling, multiple subcutaneous neurofibromas and right gluteal mass.

Blood counts and comprehensive blood biochemistry, and urine analysis confirmed an adequate end-organ function. Basal serum levels of circulating endothelial cells were performed. Baseline MRI documented a total volume of PNFs of about 1793 ml.

Imatinib Mesylate was administrated orally until the dose of 400 mg twice a day for 7 months; subsequently the treatment was discontinued for severe anaemia due to gastric bleeding (grade 3 AE). During the treatment the patients lamented mild asthenia (grade 1 AE).

Over the drug administration period, the PNFs volume increased of 4.5%.

Cognitive abilities and adaptive-social functioning remained age-appropriate during different clinical follow-up, as well as the quality of life perceived consistently good and no influenced by the general clinical condition.

12 months after discontinuation of treatment, clinical and neuroradiological stabilities was documented (growing rate 3.6% per year).

***Patient 7:*** BM, a 22-years-old female with a familial Neurofibromatosis type 1 (c.5543 T>A), was followed since infancy for café-au-lait macules, axillary and inguinal freckling and a progressive and severe scoliosis due to multiple spinal neurofibromas and wide lumbar and sacral paraspinal PNF.

Pre-treatment physical examination revealed the presence of diffuse café-au-lait macules, inguinal and axillary freckling, multiple subcutaneous neurofibromas and a severe right convex scoliosis.

Blood counts and comprehensive blood biochemistry, and urine analysis confirmed an adequate end-organ function; basal serum levels of circulating endothelial cells were analysed. Baseline MRI documented a total volume of PNFs of about 2030 ml.

Treatment with Imatinib Mesylate was administrated orally at the dose of 400 mg twice a day only for 1 months; subsequently the treatment was administered at a lower dosage (Glivec 300 mg x 2) due to nausea, loss of appetite, thrombocytopenia, anaemia and leucopoenia (all grade 1 AV) for 6 months. Then the treatment was discontinued for a right knee arthrosynovitis (grade 2 AE).

Over the drug administration period, the PNFs volume increased of 3%.

Cognitive abilities remained age-appropriate during the follow-up. And quality of life was perceived consistently good and no influenced by the pharmacological treatment, with high levels of tolerance and adaptability to the disease.

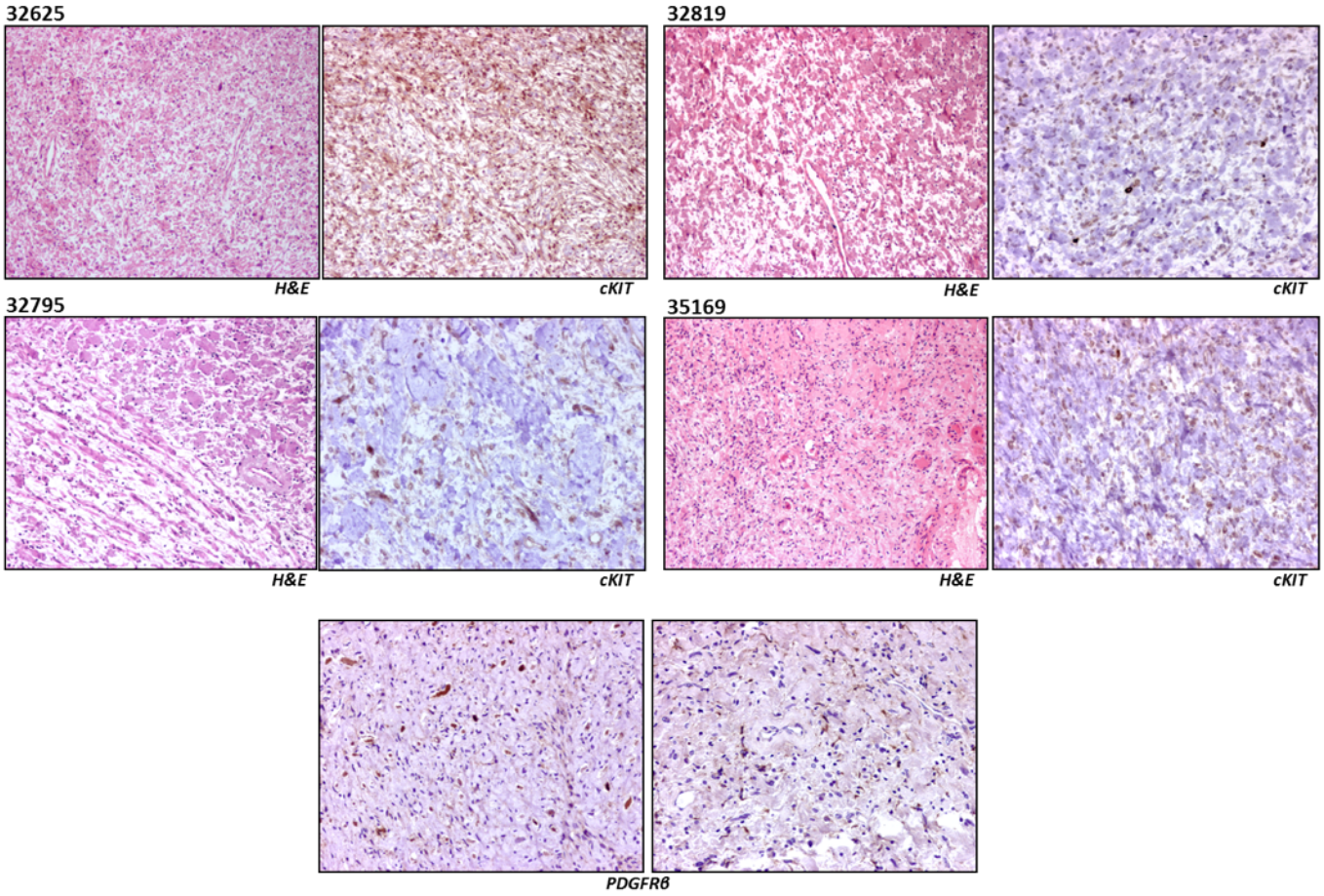
12 months after discontinuation of treatment, clinical and neuroradiological stabilities was documented (growing rate 4.3% per year).

### *5.2 Response to Imatinib Mesylate Tretament*

7 patients (4 females and 3 males) were enrolled in this longitudinal follow-up clinical trial, approved by the Italian Ministry of Health. The study allowed the inclusion of 6 NF1 patients and 1 child (n.3) within a compassionate use program, with diffuse and infiltrative PNFs with primary paraspinal and trunk/abdomen and lower-extremities extension. The

age ranged 5-35 years (median age 17 years) and the median baseline volume was 3568 ml (range 2030-6683 ml).

Analysis of c-KIT and PDGFRβ on PNFs patients' specimens revealed an expression ranging from 10 to 50% and >10% respectively in all the sample.

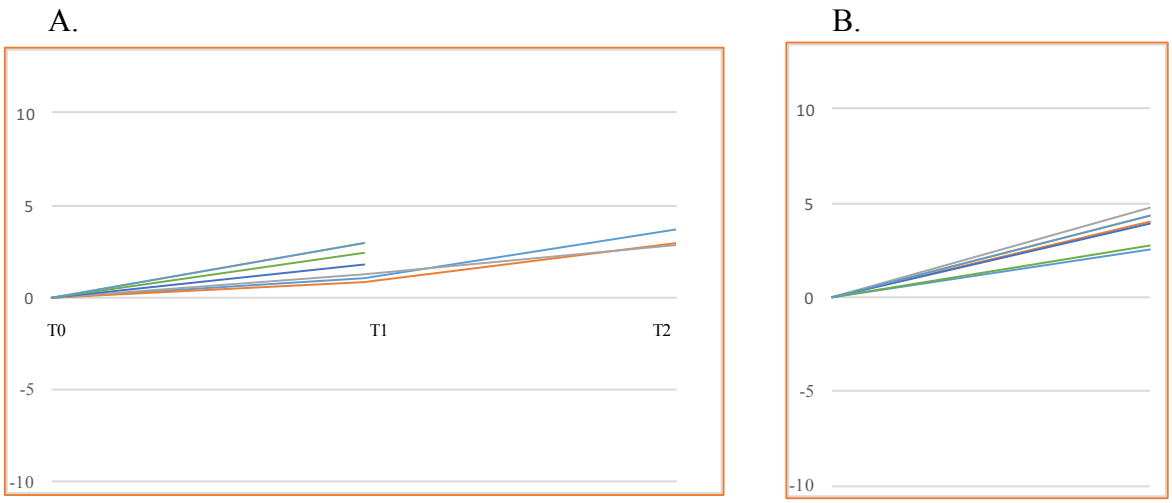



*The figure shows in the upper part examples of H&E staining and immunohistochemical analysis of cKIT and in the lower part the PDGFRβ expression (Magnification H&E and PDGFRβ 10X; cKIT 20X).*

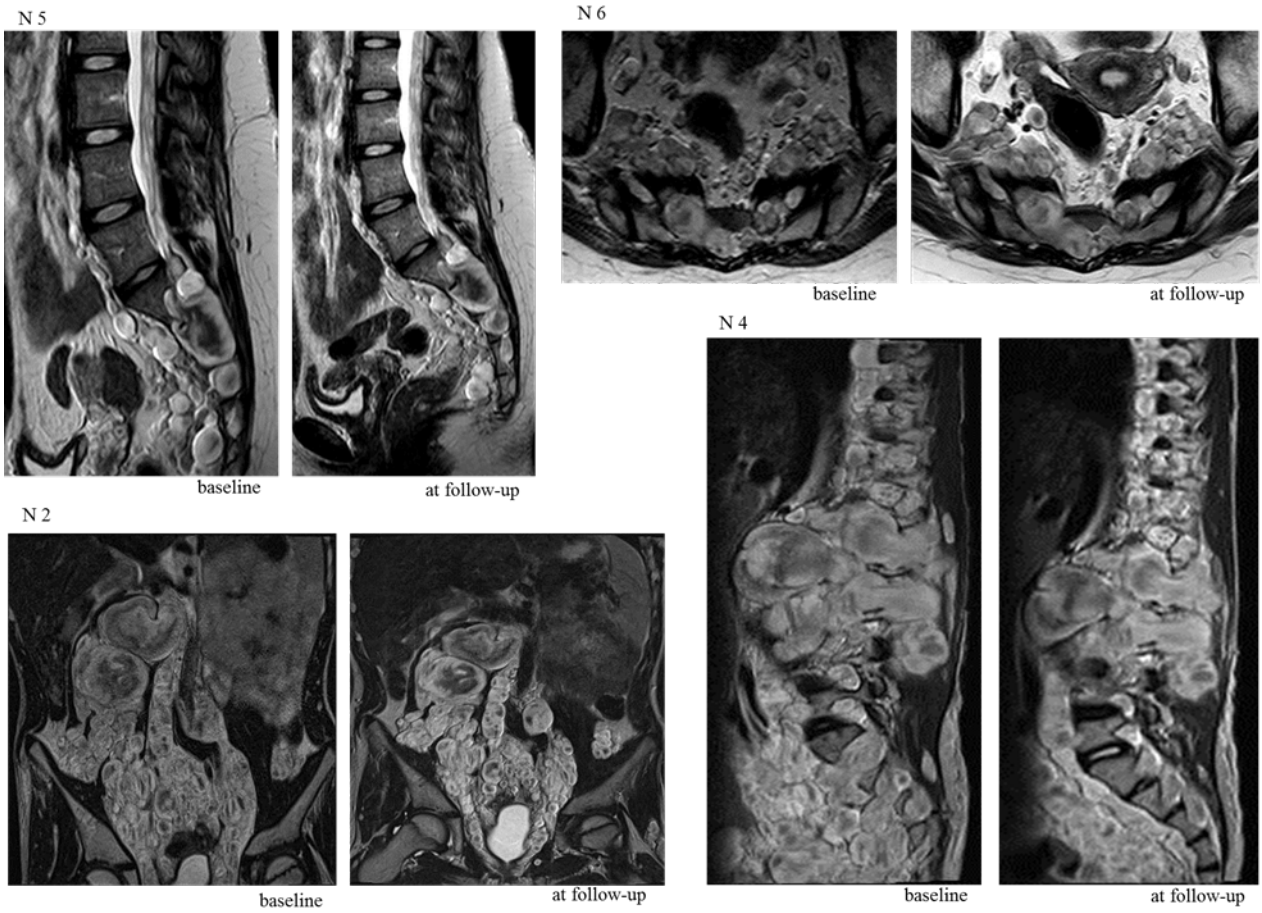
Only 2 patients completed the 12-month treatment with an adequate dosage (n.1-2); in patient n.3 IM was administered during the entire period but at a lower dosage due to drug side effects. The remaining 4 subjects withdrew from the study prematurely because of severe side effects (n. 6-7) and other site tumours progression (n. 4-5).

In particular, about Imatinib Mesylate toxicity, severe adverse events (grade 2-3 AE) included: grade 3 diffuse oedema, weight gain and renal failure in patient 3 who already presented a chronic renal insufficiency; grade 2 arthrosynovitis (n.7) and grade 3 anaemia and gastric bleeding (n.6). In addition, grade 2-3 paraesthesia and grade 1 nausea, loss of appetite, thrombocytopenia, anaemia, leucopoenia, asthenia were reported, reversible in all the cases. Notable, children had minor IM toxicity rather than adult patients.

None patients had an objective response to Imatinib Mesylate, defined as at a least a 20% decreased in tumour volume, but a substantial stability in plexiform neurofibromas volume with a median growing rate of 1,2% (ranging from 0.8 to 4.5%) during the drug administration. After 12 months of the trial discontinuation, the PNFs growth was constant and comparable to that observed during treatment for each patient (median 3.5%; ranging from 2.5 to 4.8%).

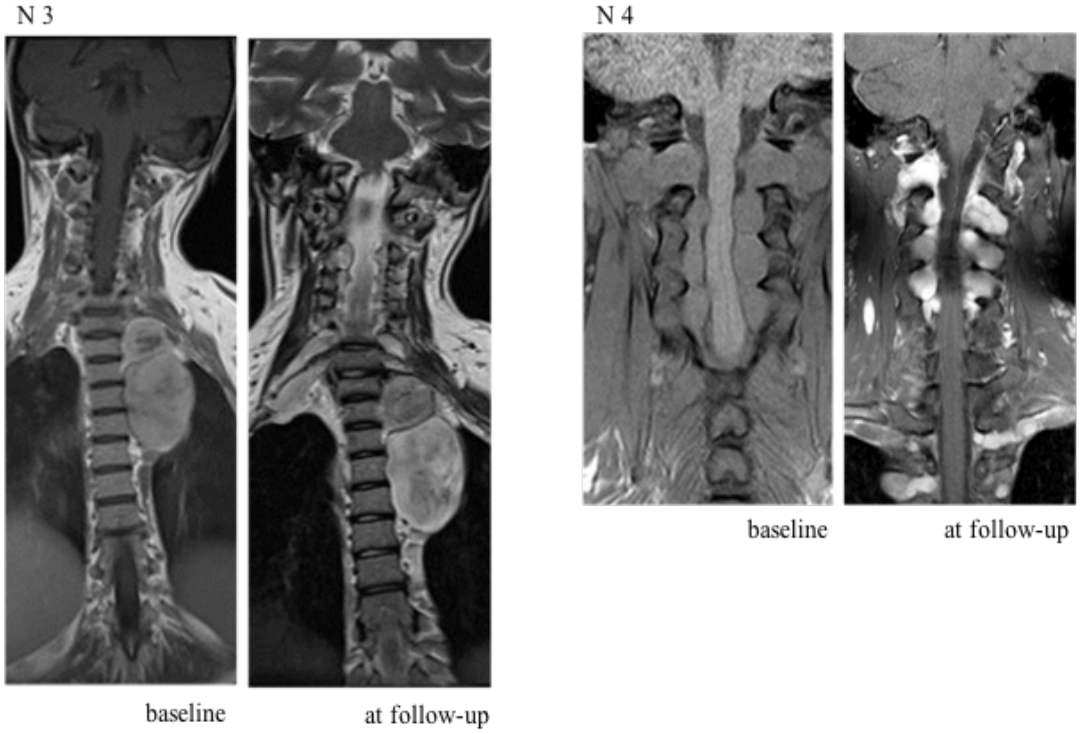



*Change in volume (%) of PNFs during treatment (A) and 12 months after drug discontinuation (B)*




*Samples of PNFs imaging at baseline and during the follow-up*

Notably, every patient presented a severe NF1 oncological phenotype with multiple further central and peripheral nervous system tumours including multiple spinal neurofibromas (n. 2-3-4-6-7), optic pathway glioma (n. 3-5) and MPNST (n. 3-4) in addition to PNFs. During the pharmacological treatment, clinical course worsened in 2 subjects due to malignant progression into MPSNT (n. 3-4) and evolution of a cervical neurofibromas compressing the spinal cord in another one (n. 5).




*Progression of atypical neurofibromas in MPNST (N 3) and compression of spinal cord (N 4)*

The evaluation of cognitive abilities and the administration of age specific Health-related quality of life scales, documented no significant change in the mean total scores from baseline to treatment course.

The characteristics of the sample and the toxic and data response are summarized in the following table.

| | |
|---|---|
| **Patient** | |
| Age at study entry | 17 years (5-35 y) |
| < 18 years | 4 |
| >18 years | 3 |
| Sex | |
| Male | 3 |
| Female | 4 |
| Median performance status score (range) | 70 (60-90) |
| **Plexiform Neurofibromas** | |
| Median baseline tumour volume (ml) | 3568 ml (2030-6683) |
| Site (number of patients-no.) | |
| Paraspinal | 7 |
| Abdomen/pelvis | 5 |
| Extremity | 3 |
| Trunk | 2 |
| Progression status of target PNF at enrolment | |
| Progressive | 7 |
| Non-progressive | 0 |
| Documented PNF-related complication at baseline (no.) | |
| Disfigurement | 4 |
| Pain | 4 |
| Neurological signs | 3 |
| Previous intervention for treatment of PNF (no.) | |
| Debulking surgery | 2 |
| Medical intervention | 3 |
| **Treatment response** | |
| Median treatment duration (months) | 8.2 (4-12) |
| Median growing rate during the treatment | 1.2% ( 0.8-4.5%) |
| Median growing rate after 12 months of drug discontinuation | 3.5% (2.5-4.8%) |
| Adverse event (numbers of events) | |
| grade 1 | 6 |
| grade 2 | 2 |
| grade 3 | 6 |
| Reason to discontinue drug or dose adjustment | |
| other site atypical neurofibromas progression | 2 |
| grade 2-3 adverse events | 3 |

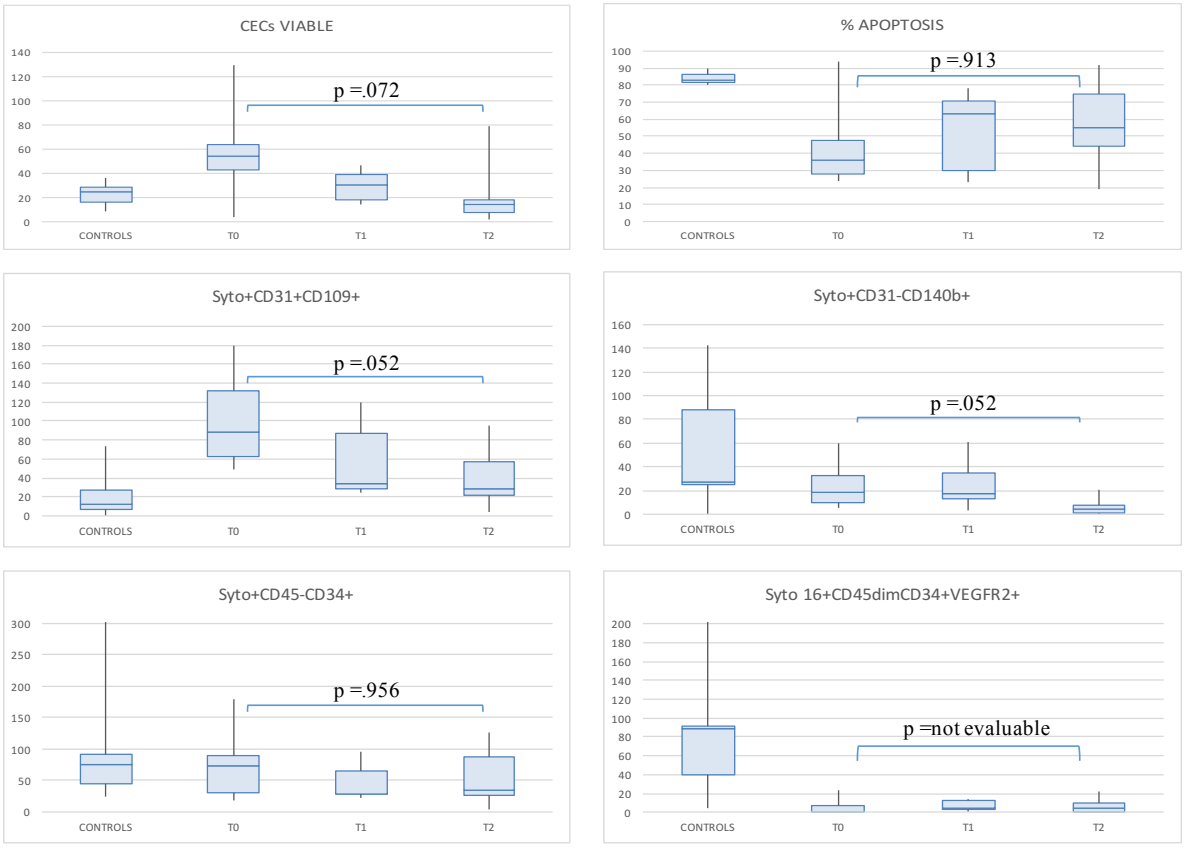
*Patient characteristics, response and toxicity data*

The investigation of angiogenic factors (circulating endothelial cells and progenitors), revealed higher levels of viable CECs (Z=-2.111; exact p=.012), percentage of apoptosis (Z=-2.241; exact p=.038), and CD109+ (Z=-2.747; exact p=.002) and VEGFR2+ hematopoietic progenitors (Z=-2.649; exact p=.007) at baseline, comparing to healthy controls. Conversely, no statistically significant difference was noted in CD140b (Z=-.958; exact p=.383) and EPC (Z=-.575; exact p=.620) levels.

During the treatment, we observed:

- a statistically significant reduction of CD109+ CECs ($\chi^2$= 6.333; exact p= .052), CD140b+ PPC ($\chi^2$= 6.333; exact p= .052);
- no statistically significant reduction of apoptosis percentage ($\chi^2$= .261; exact p= .913), CD34+ EPCs ($\chi^2$= .333exact p= .956), VEGFR2+ (not evaluable);
- a result close to statistical significance in vital CECs ($\chi^2$= 5.333; exact p= .072) reduction.

Furthermore, no significant relationship was observed between biological markers and clinical or radiological severity using non parametric Spearman correlation (p=.470 and p=.510 respectively).




*Variations of CECs and EPC serum level at baseline and during the follow-up*

## DISCUSSION

Plexiform neurofibromas (PNFs) are often-congenital lesions and can result in substantial morbidity and early mortality associated with Neurofibromatosis type 1.

PNFs grow at variable and unpredictable rates, particularly in childhood. These lesions can be multiple and extensive, and often have clinical consequences including severe disfigurement, pain, organ compression and other functional impairment related to infiltration, displacement and compression of the surrounding structures.[1] In addition, PNFs contribute to malignant peripheral nerve sheat tumours (MPNSTs) pathogenesis, with a lifetime risk of about 13-15% to malignant progression.

The indolent growth characteristics of plexiform neurofibromas and the potential for inducing malignant transformation to MPNSTs are significant concern limiting the use of traditional conventional cytotoxic chemotherapies in the treatment of these lesions. Thus, the development of targeted molecular therapies with the potential to induce growth arrest and/or regression of these slow-growing tumours represents a significant area of clinical morbidity.[32]

Preclinical studies in a NF1 mouse model showed reduced PNFs volume in response to the kinase inhibitor Imatinib Mesylate (IM) and established the rationale for the following clinical trial in plexiform neurofibromas.[43,45] The mouse studies were supported by a case report of a 3-year-old child with NF1 and a PNF that caused critical airway compression, treated with Imanitib resulting in a tumour volume reduction of more than 50%.[45]

On the basis of these findings, Robertson and colleagues presented the results of a phase 2 pilot study, which showed a reduction in size of symptomatic plexiform neurofibromas in patients with NF1 treated with Imanitib Mesylate administered orally.[48]

In that study, 36 NF1 patients aged 3-52 years with PNFs were enrolled and treated with daily oral Imtinib Mesylate at 220 mg/m2 twice a day in children under 19 years old, and 400 mg twice a day in adults. 23 of them, with a total of 69 tumours volumetrically measurable, completed the study drug receiving the treatment for at least 6 months while 13 patients withdrew from the study prematurely

The authors reported an objective response to Imatinib Mesylate in six of the 23 evaluable patients (26%) who received the treatment for at least six months, noting a profound response in a subset of PNFs, some of which reduced in volume by almost 40% with a median reduction of 26,5%. Although eight (12%) of 69 individual tumours exceeded the 20% volume reduction threshold constituting treatment response, only one patient continued on long-term treatment. A further 36 (52%) tumours remained stable (<20% increase or decrease in volume) and 25 (36%) progressed (≥20% increase in volume).

On the basis of the recent data reported in literature, the primary endpoint of this study was to assess whether the inhibition of c-kit signalling pathways by Imatinib Mesylate results in objective reduction and/or inhibition of the growth of progressive, surgically inoperable plexiform neurofibromas (PNFs) in NF1 patients.

Owing to the expanding understanding of the basis of PNFs development and of Imatinib-mediated tyrosine kinase inhibition,[43,49] the evaluation of c-Kit staining on tumoural specimens was mandatory to highlight recruited mast-cells. In addition, PDGF-β staining was performed to evaluate the recruitment of vascular endothelial cells.

All the 7 patients who fulfilled the eligibility criteria, presented extensive and complex PNFs, defined as lesions with blurred margins and extension or infiltration to adjacent tissues, with a primary paraspinal location and trunk/abdominal/lower extremities

extension. Median volume was of 3568 mL (range 1793-6683 mL). Notable, the selected patients for the drug trial had all a more severe oncological phenotype both for PNFs extension that for the presence of multiple central and peripheral nervous system tumours, compared to the previous studies.[45,48]

In the designed trial, the drug should have been administered orally at the dose of 220 mg/m$^2$ twice a day for children and 400 mg twice a day for adults, for 12 months unless severe toxicity. Four patients withdrew from the study prematurely (range 4-7 months): specifically, two of them had severe side effects (gastric bleeding and arthrosynovitis respectively), and the other two had evidence of additional symptomatic growing lesions despite a stable PNF. Only two patients completed 12 months of study drug with and adequate dosage (n.1-2) while in patient n.3 required a lower dosage due to renal failure.

Contrary to Robertson findings, we haven't obtained an objective response in IM treatment. Instead we documented a substantial stability in plexiform neurofibromas volume, with a growing rate ranging from 0.8 to 4.5% during the drug administration, and a constant and comparable PNFs growth even after IM suspension (ranging from 2.5 to 4.8%). Furthermore, mild to severe Imatinib toxicity was also recorded in 5 patients, with a worse drug tolerance in adult subjects respect to the younger ones.

Notably, 3 patients presented a progression of other different tumours (atypical and typical neurofibromas) despite the stability of PNFs during the Imatinib Mesylate administration. The heterogeneity in response for different lesions in the same patient confirmed the complexity of NF1-related tumours development and the presence of different pathogenic processes arising as genetically distinct primary tumours in a single individual.

The first critical observation about the results of our trial was that all the patients who came off the study did not take the drug long enough to test the biological effects. On the other hand, as previous reported, larger and not head/neck located tumours tended to be less responsive to the pharmacological treatment, perhaps indicating difficulty in drug delivery into large solid lesions.[48] Indeed, volume decreases of at least 20% were limited to small tumours of less than 20 ml,[48] which were substantially smaller than all the PNFs evaluated in our trial.

Additionally, untreated PNFs never regress, but rather display variable progressive growth with a continued increase in tumour volume over time by a variable rate from 3.1 to 68.3% (median of 14.3%) per year. [57] A faster growth speed exceeding the increase in their BMI was also reported in younger individuals aged under 13 than in older ones (2 $cm^2$/year and < 1$cm^2$/year respectively).[58] However, these few studies on the natural history of PNFs highlighted an alternation of unpredictable periods of growth and stability; in view of these findings, the sustained stability of tumour volume over time in all of our patients could not be consistent with a treatment response.

Consistently with this consideration and supported by the results of a phase I preliminary study,[59] recently Jackacki et al. conducted a phase II trial evaluating both clinical and imaging response rate to administration of Pegylate Interferon (PI), an interferon with a significantly prolonged plasma half-life and with an antitumor activity that is achieved through direct antiproliferative and cytotoxic effects, modulation of the host immune and inhibition of angiogenesis.[60]

The authors underlined that if only radiological response had been used as the primary endpoint, the significant impact of various pharmacological treatment, including PI, would

have been both minimal and controversial in evaluating outcome of PNFs patients, since the natural history of these tumours is characterized by an unpredictable growing rate over the time.

Thus, in the PI trial a specific stratum of 26 patients with formally documented symptoms was introduced to objectively evaluate clinical improvement. The symptoms included loss of function due to plessiform neurofibromas, reflected by a decrease in Karnofsky or Lansky score by >1 level within the year preceding trial entry, and PNF-associated pain requiring daily pain medication. Clinical response was defined as an improvement of at least one level in performance status, >50% decrease in the amount of pain medications required per week compared with baseline, or ability to change from a narcotic to a nonnarcotic analgesic.

Only 4 of the 26 enrolled patients (15.4%) had a clinical or radiological response, although clinical improvement did not correlate with radiographic changes in symptomatic patients.

Even if the efficacy of Pegylate Interferon was proven lower than it was reported in the phase I trial, it is interesting to note that the study considered the clinical objective response, rather than the radiological one, as one of the primary endpoints.

In our trial, the potential clinical response to treatment with Imatinib Mesylate was not assessed prospectively. However, the absence of significant changes in the mean total scores of specific Health-related quality of life scales, or in the functional status according to Karnofsky or Lansky performance scale from baseline to treatment course, suggests the lack of both subjective and objective clinical benefits.

It is noteworthy that, more recently, the availability of other agents that target RAS signalling and other pathways implied in the pathogenesis of plexiform neurofibromas has

led to another promising study with Selumetinib, an oral selective MEK 1-2 inhibitor that in the neurofibroma animal model showed, among all of the pharmacological treatments that were tested, the greatest activity.[61]

In this phase I trial, all the 24 children with inoperable plexiform neurofibroma benefited from some decrease in tumour volume and clinical improvement, and none suffered from disease progression.

Obviously, the positive results obtained with this drug will have to be confirmed by phase 2 trials, but still they are very encouraging.

However, independently of these results, all these studies demostrate that we are heading to a new and promising era in which, looking for agents that target the growth control pathways regulated by the NF1 gene, we may be able to treat the underlying cause of the disease.

The second aim of this study was to identified potentially predictive and prognostic biological markers of response to treatment or of tumour progression.

Recent research focused on the necessity of discovering biomarkers useful for early prediction of tumours, evaluation of response to a treatment and forecasting progression or recurrence.[50]

For Neurofibromatosis type 1, all biomarkers published to date are considered exploratory and none have been validated. Furthermore, most of the NF1 research focused on complex molecular predictive biomarkers and candidate gene alterations. The opportunity to study novel agents in the disease requires the developing and incorporating pharmacodynamic and predictive serum biomarker studies into early clinical trials.

In relation to the Imatinib Mesylate mechanism of action and the importance of angiogenesis in tumours pathogenesis and progression, circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) were evaluated as possible candidates to serve as such markers.

Consistently with the literature,[51,62] at baseline high level of viable CECs (Z=-2.111; exact p=.012), and VEGFR2+ hematopoietic progenitors (Z=-2.649; exact p=.007) compared to healthy control were found, mirroring an aberrant vascular turnover/remodeling process in patients with tumours.

Conversely, no statistically significant difference was noted in CD140b (Z=-.958; exact p=.383) and EPC (Z=-.575; exact p=.620) levels when comparing patients and healthy control groups. However, an increase in EPCs is correlated with promotion of metastasis and typical PNFs such as grade I tumors have a low metastasis potential until they become malignant. [51]

Furthermore, CD140b represents an essential component of the microvessels wall, that is associated mainly with stabilization and hemodynamic processes involving blood vessels in a manner that depends on tissue and angiogenic stage. Infact, pericyte density is tissue specific and the higher density found in the vessels of the brain justifies the increase in seric levels of these biomarkers which occurs in case of brain tumours rather than in the peripheral ones.[62]

During the treatment, results close to statistical significance in CD109+ CECs (exact p= .052), CD140b+ PPC (exact p= .052), and vital CECs (exact p= .072) reductions were noted. These findings are consistent with the anti-angiogenetic mechanism of action of

Imatinib Mesylate by PDGFR-related inhibition of the signaling pathway of molecules involved in angiogenesis.

Since no significant relationship was observed between the variations in seric levels of angiogenic factors during the treatment and clinical or radiological severity and evolution, CECs and EPCs seem to be indicators of antioangiogenetic Imatinib Mesylate activity rather than useful biomarkers for predicting response or forecasting progression in PNFs patients. Thus, other main biological programs may be involved in PNFs development.

Nevertheless, it is important to notice that, in our study, all of the patients had similar responses to treatment, so it was not possible to evaluate the variations of CECs and EPCs between responders and non-responders. In addition, the studied sample is small and close to the limit of statistical significance itself.

Further studies are required to determine the exact role that each cell plays and whether CECs and EPCs may be suitable as response indicators for the tyrosine Kinase inhibitor treatment.

## CONCLUSION

The present clinical trial with Imatinib Mesylate highlighted that patients with extensive and complex PNFs with a primary paraspinal location have not an objective response to the treatment;

furthermore, the evolution of other tumours despite the stability of PNFs during the Imatinib Mesylate administration confirmed the complexity of NF1-related tumours development and the presence of different pathogenic processes, reflecting in genetically distinct primary tumours in the same individual;

CECs and EPCs seems to be indicators of anti-angiogenetic Imatinib Mesylate activities rather than useful biomarkers for predicting response or forecasting progression.

This preliminary research requires larger studies in order to profile more precisely which Neurofibromatosis Type 1 patients with plexiform neurofibromas may benefit from Imatinib Mesylate, and to indentify factors underlying the variability of treatment response to different candidate medications. Moreover, considering that current knowledge of pathogenetic mechanism leading to PNFs formation is increasingly deep and accurate, other target drugs could be screened for activity against plexiform neurofibromas.

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