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XXXVI CICLO DEL DOTTORATO DI RICERCA IN SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

Precision Medicine and Target Therapy on Rare Diseases: the case of Neurofibromatosis type 1. Clinical data and transcriptome analysis

Settore scientifico-disciplinare: MED/38 PEDIATRIA GENERALE E SPECIALISTICA

DOTTORANDO
ANDREA MAGNOLATO

COORDINATORE
PROF. PAOLO GASPARINI

SUPERVISORE DI TESI
DOTT.SSA IRENE BRUNO

CO-SUPERVISORE DI TESI
PROF. EGIDIO BARBI

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INTRODUCTION

Neurofibromatosis Type 1 (NF1), also known as von Recklinghausen's disease, is a relatively common genetic disorder that affects the nervous system and various other parts of the body. It is also classified as a multisystem disorder because of the clinical diverse manifestations including neurocognitive, skeletal and cardiovascular abnormalities, and is also classified as a neurocutaneous syndrome to highlight its manifestations primarily in the nervous system and the skin as a result of lesions affecting neural-crest-derived tissues such as Schwann cells and melanocytes. It is characterized by the development of noncancerous tumors, called neurofibromas, along nerves and other skin abnormalities. NF1 is an autosomal dominant genetic condition, which means that a person can inherit the disorder from one affected parent. This disorder can manifest in various ways and can have a wide range of symptoms, making it a complex condition to manage and understand. In this introduction, we will delve into the key aspects of NF1, including its prevalence, clinical manifestations, genetic basis, and potential complications

Incidence and prevalence

Although traditionally considered a rare disease, NF1 is relatively common, with an estimated prevalence of around 1 in 3,000 individuals. In recent European population studies the incidence of NF1 is approximately 1 in 2000 to 1 in 3000. NF1 is fully penetrant in adults, but many disease features increase in frequency or severity with age. The European studies also demonstrated that NF1 can be diagnosed by age 6 years in most cases (95%) by routine physical examination with special attention to the disease-associated skin stigmata[1,2]. There are no known ethnic groups in which NF1 does not occur or is unusually common. The prevalence is somewhat higher in young children than in adults, difference that probably results at least in part from the early death of some NF1 patients[3,4].

Pathogenesis

Neurofibromatosis type 1 is caused by mutations that occur in the NF1 gene, at chromosome 17q11.2. NF1 encodes neurofibromin, a ubiquitous protein mainly expressed in neurons, Schwann cells, and glial cells[5]. Neurofibromin is a multifunctional protein capable of regulating multiple signaling pathways including Ras/MAPK[6], Raf/MEK/ERK[7], PI3K/AKT/mTOR[8], Rho/ROCK/LIMK2/cofilin[9], PKA-Ena/VASP[10] and cAMP/PKA[11]. The NF1 is today included in the group of RASopathies due to neurofibromin-mediated inactivation of the RAS/MAPK pathway (fig. 1). This pathway is responsible of proliferation, differentiation, cell migration, and apoptosis[12]. RASopathies also include Noonan (MIM 163950), LEOPARD (MIM 151100), Costello (MIM 218040), Cardio-facio-cutaneous (MIM 115150), and Legius (LGSS; MIM 611431) syndromes. All these disorders share several phenotypic characteristics (e.g., skin involvement, neurocognitive impairment, dysmorphisms, increased oncological risk), in some cases making differential clinical diagnosis complex[13].

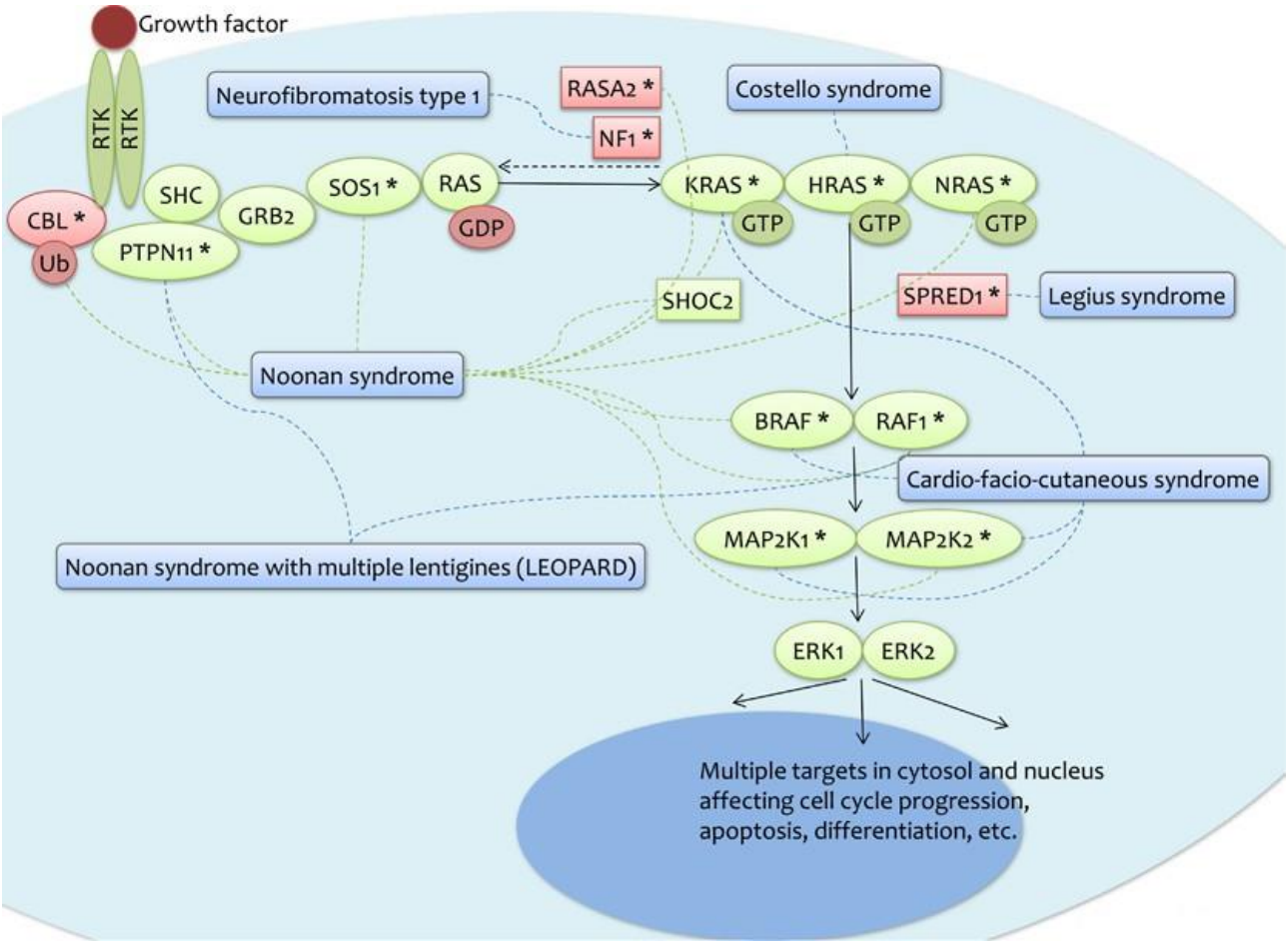

Fig.1 Rasopathies and RAS/MAPK pathway[14]

It was recently found that neurofibromin exists as a constitutive high affinity dimer in either an "off" or an "on" configuration; in the "on" conformation neurofibromin can interact with RAS. The neurofibromin GAP-related domain negatively regulates RAS by increasing its intrinsic GTPase activity facilitating the conversion of active RAS-GTP to inactive RAS-GDP[15,16]. Only the neurofibromin dimer in its "on" configuration can interact with RAS. Lack of functional neurofibromin in patients with NF1 leads to dysregulated RAS and tumorigenesis (fig. 2).

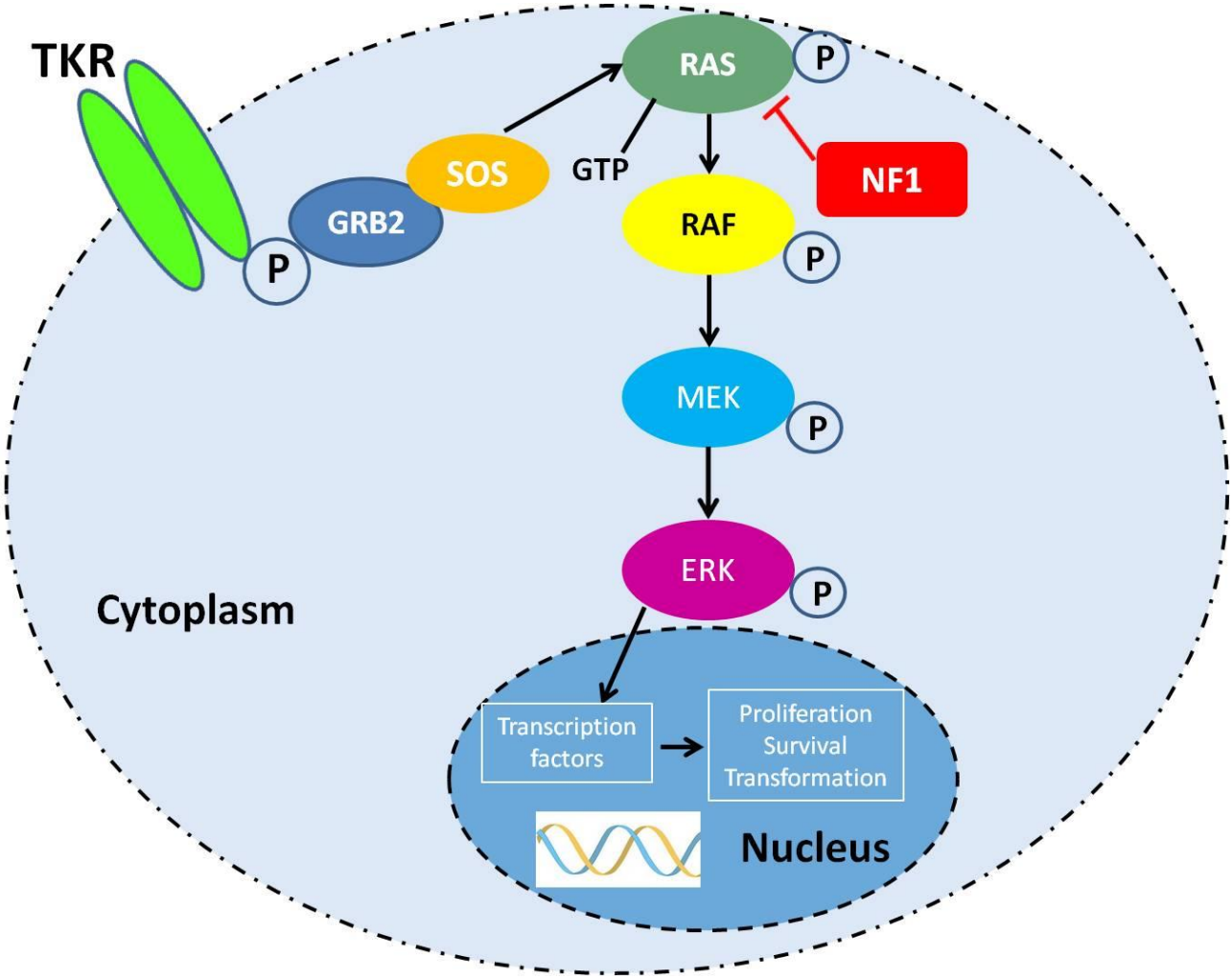

Fig. 2 Integration of the MAPK pathways in the cellular response to various environmental stimuli. The sequential phosphorilation of RAS-RAF-MEK ERK, after dimerization of TKR, and the role of negative regulation of NF1[17]

Diagnostic criteria

The modern history of nomenclature of neurofibromatosis started in 1987 with the National Institutes of Health (NIH) Consensus Development Conference on Neurofibromatosis[18]. The NIH criteria include the most frequent disease manifestations (CALMs, freckling, neurofibromas, and Lisch nodules) alongside disease complications typical of NF1 (fig. 3).

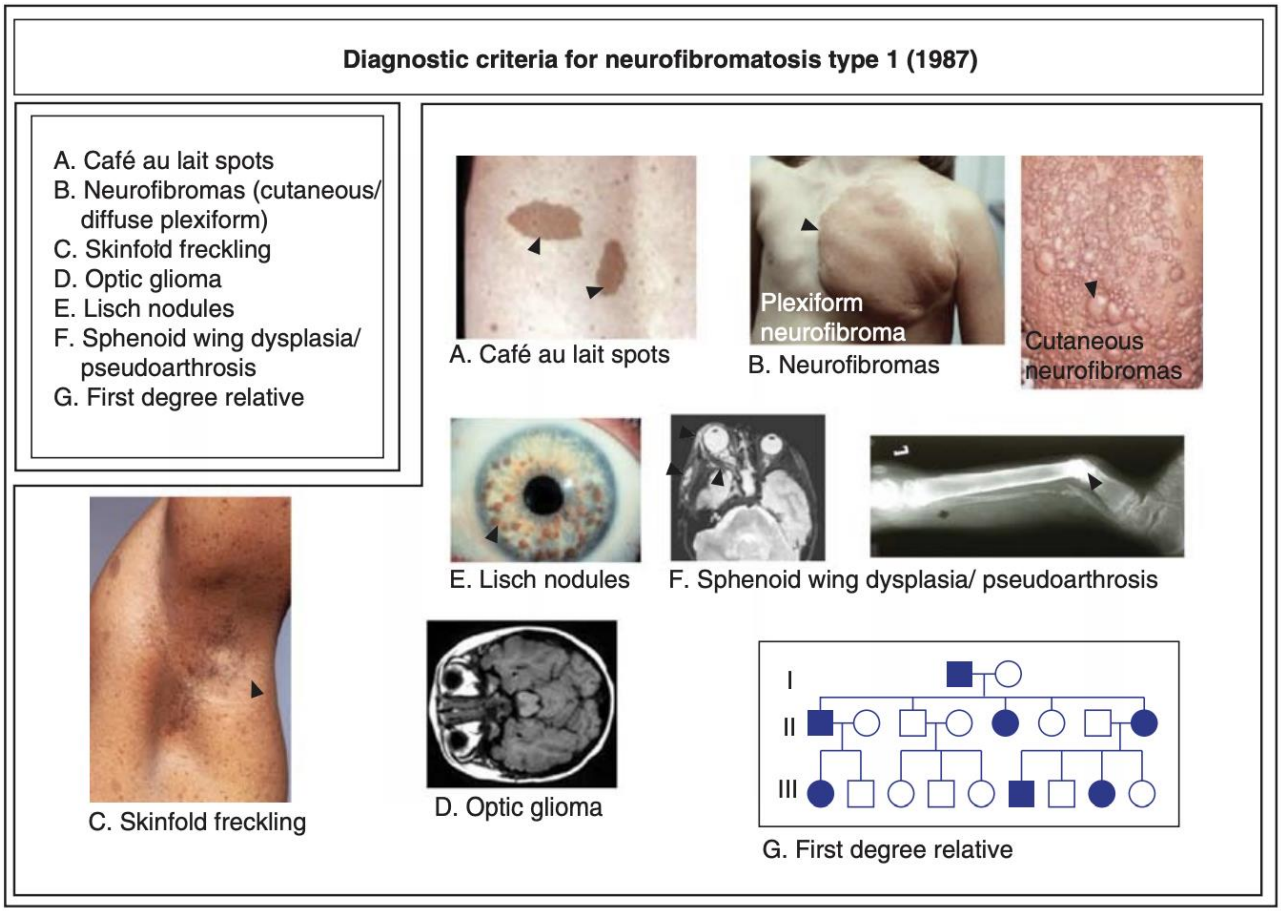

Fig. 3 NIH diagnostic criteria for NF1. A. Six or more CAL > 5 mm in children and > 15 mm in postpubertal individuals; B. two or more neurofibromas of any type or one plexiform neurofibroma; C. axillary and/or inguinal freckling; D. optic glioma; E. two or more Lisch nodules; F. sphenoid wing dysplasia or pseudarthrosis; G. a first-degree relative with NF1.[19]

Since 1987 there has been one formal review of the NF1 criteria by the Clinical Care Advisory Board of the National Neurofibromatosis Foundation (now the Children's Tumor Foundation)[20]. No criteria alterations were suggested.

In the 2021 a consensus panel guided by Legius revised criteria for NF1 incorporating new clinical features and genetic testing[21] (table 1). The objective was to update the criteria by integrating new

clinical and genetic findings since the initial consensus conference. They employed a modified Delphi method to achieve consensus among stakeholders, acknowledging that complete agreement might not be feasible. Throughout the process, a recurring challenge was finding a balance between the expectations of different medical specialties regarding diagnostic criteria. For instance, some specialists stressed the importance of early diagnosis in children to detect crucial medical features (high sensitivity), while others emphasized the need to prevent potential misdiagnoses (high specificity). Ultimately, the group aimed to select criteria that struck a balance between high sensitivity and specificity.

Table 1. Revised diagnostic criteria for neurofibromatosis type 1 (NF1).

A: The diagnostic criteria for NF1 are met in an individual who does not have a parent diagnosed with NF1 if two or more of the following are present:

- Six or more café-au-lait macules over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals[a] (Supplementary Fig. 6)
- Freckling in the axillary or inguinal region[a] (Supplementary Fig. 7)
- Two or more neurofibromas of any type *or* one plexiform neurofibroma (Supplementary Fig. 8a, b)
- Optic pathway glioma (Supplementary Fig. 9)
- Two or more iris Lisch nodules identified by slit lamp examination or two or more choroidal abnormalities (CAs)—defined as bright, patchy nodules imaged by optical coherence tomography (OCT)/near-infrared reflectance (NIR) imaging (Supplementary Fig. 10a, b)
- A distinctive osseous lesion such as sphenoid dysplasia,[b] anterolateral bowing of the tibia, or pseudarthrosis of a long bone (Supplementary Fig. 11)
- A heterozygous pathogenic *NF1* variant with a variant allele fraction of 50% in apparently normal tissue such as white blood cells

B: A child of a parent who meets the diagnostic criteria specified in A merits a diagnosis of NF1 if one or more of the criteria in A are present

[a]If only café-au-lait macules and freckling are present, the diagnosis is most likely NF1 but exceptionally the person might have another diagnosis such as Legius syndrome. At least one of the two pigmentary findings (café-au-lait macules or freckling) should be bilateral.
[b]Sphenoid wing dysplasia is not a separate criterion in case of an ipsilateral orbital plexiform neurofibroma.

Table 1: Revised diagnostic criteria for Neurofibromatosis type 1[21]

Clinical Manifestations

NF1 is a highly variable multisystem disease. The progression and severity can vary over an individual's lifetime as well as among family members who have NF1 pathogenic variant[22,23].

Cutaneous features which are readily apparent on visual inspection, are usually the first sign of the disease. The largest and best known cutaneous manifestation in NF1 are the Café au lait macules (CALMs), which are in general typically flat, uniformly hyperpigmented macules with regular, well-defined borders. They may be smaller or much larger, lighter or darker and sometimes irregular in shape. They usually appear in infancy and early childhood, and once established remain stable in number and size (except for milli metrical growth of the skin itself). The occurrence of CALMs appears to be stochastically dispersed in the skin[24]. Histologically, they have increased melanin in melanocytes and basal keratinocytes, but no melanocyte proliferation. CALMs appear flat and have the same level as the surrounding skin. If a lesion has raised skin or an unusually soft or uneven texture compared to the surrounding skin, it's likely an indication of an underlying plexiform neurofibroma. The darker pigmentation of CALMs may be challenging to discern in individuals with very fair or very dark skin, where the color of the lesions closely matches that of the surrounding skin. In such cases, a Wood's light can be helpful in highlighting the pigmented macules (fig. 4A). Freckling, also known traditionally as the Crowe sign, refers to the presence of small pigmented lesions (measuring 1 or 2 mm) with a light brown color. These freckles typically do not appear at birth but develop during childhood, usually from the age of 2 onwards. Their appearance is similar to freckles induced by sun exposure, but in NF1, they typically occur in regions with minimal to no sun exposure. They are commonly found in specific areas of the body, such as the axillae, inguinal regions, areas of skin under the breasts in women, the base of the neck, and the upper eyelids. These locations are believed to be influenced by various physical factors like increased skin temperature, limited exposure to light, and the presence of skin secretions such as sweat[25,26](fig. 4 B).

Neurofibromas are benign tumors made up of Schwann cells and can affect almost any nerve in the body[27]. Cutaneous neurofibromas are well-defined masses found on the skin, typically ranging in size from 1-2 mm to a few centimeters. They can have various textures, from soft to rubbery to firm. These growths may be flat or raised, and the affected skin can either match the color of nearby unaffected skin or appear slightly pinker, browner, or bluer. Most of them don't cause any symptoms, but some can itch or be sensitive to touch. While cutaneous neurofibromas are rare in children, they are almost always present in adults with NF1. Subcutaneous neurofibromas are located beneath the skin and can be moved under the skin. They usually feel rubbery and form lumps, although some may be less well-defined with soft or irregular textures. The skin overlying a superficial diffuse

neurofibroma may display unusual pigmentation or hair patterns. Subcutaneous neurofibromas can occur individually, in clusters, or continuously along a nerve, resembling beads on a string. Most of them are small, but they can grow to be over 5 cm in diameter. Some subcutaneous neurofibromas can be tender and occasionally cause pain. These types of neurofibromas are less common in children but are present in around 15% of adults with NF1 upon clinical examination. Both cutaneous and subcutaneous neurofibromas continue to develop throughout a person's life, although the rate at which they appear can vary significantly from year to year. The total number of neurofibromas observed during clinical examinations in adults with NF1 can range from a few to hundreds or even thousands. Some women may experience a rapid increase in the number and size of neurofibromas during pregnancy, but this does not seem to lead to a persistent increase in the overall tumor burden when compared to women with NF1 of child-bearing age who have not been pregnant[28](fig 4C).

Juvenile xanthogranuloma (JXG) is a benign, self-involuting form of non-Langerhans cell histiocytosis, consisting of an asymptomatic yellow, orange, or reddish papule or nodule, most commonly affecting the head and neck and typically occurring in young children. Although its actual incidence in the general population is difficult to determine due to its spontaneous regression over a few years, it has frequently been reported in children with NF1 and some authors proposed to adopt JXG as an adjunctive NF1 diagnostic criterion[29].

Nevus anemicus is a congenital skin disorder characterized by hypopigmented, confluent, and mottled macules resembling a grape cluster, sometimes associated with smaller satellite macules.7 Rubbing or warming induces reactive erythema in the surrounding skin, which contrasts with the persistent pallor of the Nevus anemicus. It has a high prevalence in NF1, particularly in young children. Nevus anemicus does not seem to be associated with a particular genotype of NF1 or with neuro-ophthalmologic findings such as optic glioma. It may be a helpful clinical sign to distinguish neurofibromatosis type 1 from genodermatoses with overlapping phenotypic features, particularly Legius syndrome[30] (fig. 4D).

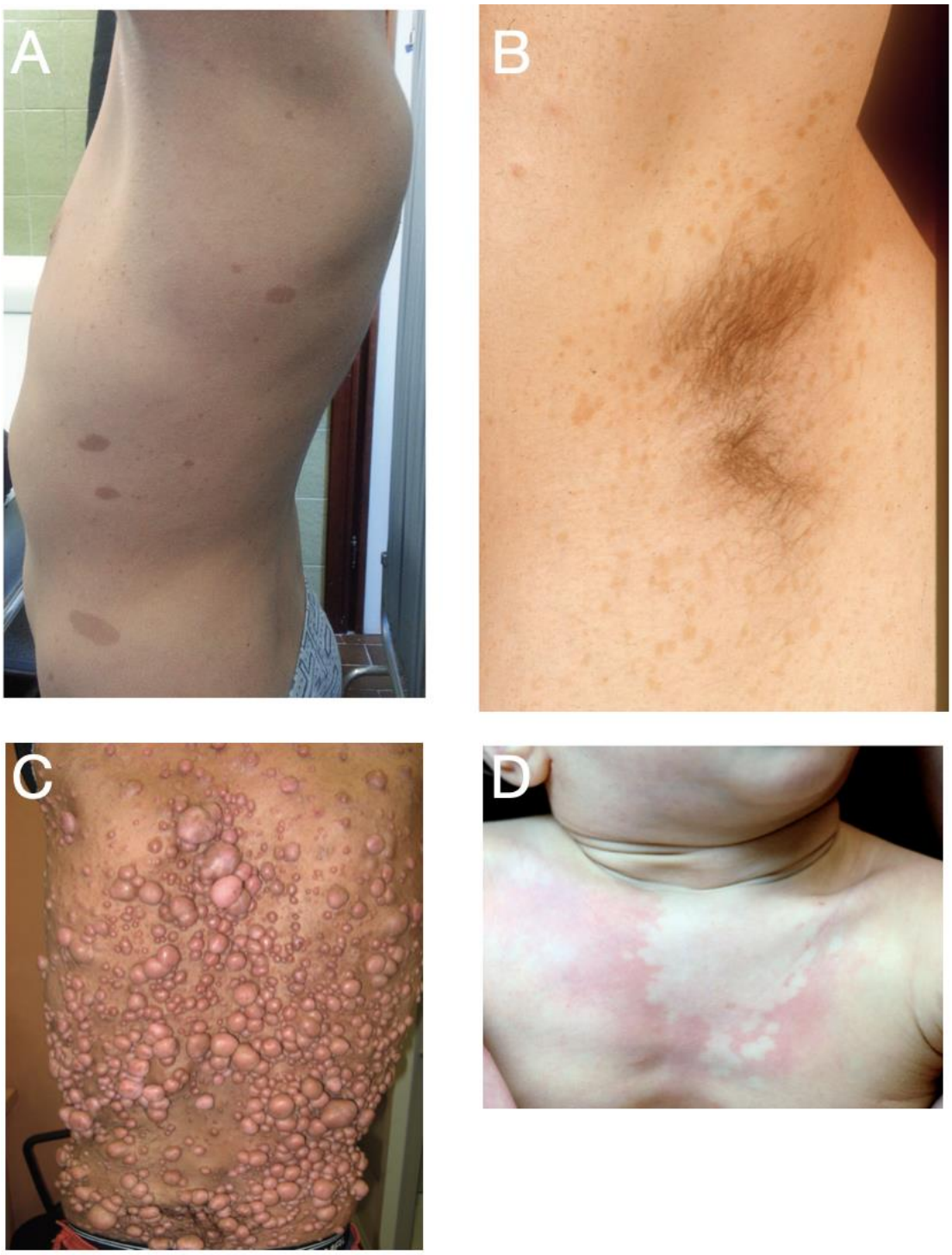

Fig.4 Cutaneous manifestation in NF1: CALMs (A), freckling (B), Neurofibroma (C) and Nevus anemicus (D)[25,30].

Ocular findings consists in Lisch nodules and choroidal freckling[31]. Lisch nodules are harmless iris abnormalities that can be observed during a slit lamp examination in nearly all adults with NF1. However, they are visible in less than half of children with NF1 under the age of five. They can be distinguished from iris freckles by their three-dimensional, nodular appearance.

Choroidal freckling cannot be detected through a standard eye examination but can be visualized using scanning laser ophthalmoscopy with infrared or near-infrared light, infrared reflectance imaging, or optical coherence tomography. These lesions consist of Schwann cell overgrowths arranged in concentric rings around an axon. They are present in the majority of individuals with NF1, regardless of age, and their prevalence, number, and size tend to increase as children grow older[32].

Recently hyperpigmented spots of the fundus oculi has been associated with NF1[33]. They are probably the same type of choroidal nodules lesion and they are not a negative prognostic factor of the disease.

Optic pathway gliomas (OPG) in individuals with NF1 are typically without symptoms, and this often remains the case throughout their lives[34]. The progression of OPG in individuals with NF1 tends to be milder than in those without NF1. In NF1, these gliomas often remain stable for many years or progress very slowly. Furthermore, it appears that a significant number of optic pathway gliomas in NF1 individuals may regress spontaneously, with their prevalence decreasing from around 20% in young children to less than 5% in older adults with NF1. Symptomatic OPG in individuals with NF1 typically manifest before the age of six, resulting in issues like decreased visual acuity, proptosis, or strabismus. However, in some cases, these tumors may not become symptomatic until later in childhood or adulthood[35]. While increased tortuosity of the optic nerve can be observed on brain MRI in children with NF1, this condition is not associated with the development of OPG in individuals with NF1[36].

Patients with NF1 develop some neoplasms more frequently and at a younger age compared with individuals without NF1[37]. Despite MPNST which will be discussed later, brain tumor, breast cancer, hematologic malignancies and sarcomas are described.

Non-optic gliomas in individuals with NF1 are often without symptoms and are typically discovered incidentally during routine head MRI screenings or for other medical reasons. These tumors are usually low-grade, slow-growing, or even non-progressive over many years. However, occasionally symptomatic and high-grade brain tumors can be observed in some cases[38]. At least 20% of individuals with NF1 who have one non-optic glioma develop two or more of these tumors. Second central nervous system (CNS) gliomas are found in 17% to 20% of individuals with NF1 who already have optic pathway gliomas.

Women with NF1 have a significantly elevated risk of developing breast cancer before the age of 50 and have a higher mortality rate from breast cancer. They also have a greater cumulative risk of developing breast cancer in the opposite breast. Breast cancers in women with NF1 are more likely to be HER2-positive and exhibit other unfavorable tumor markers[39].

While still rare, juvenile myelomonocytic leukemia (JMML) is hundreds of times more common in children with NF1 than in the general population. JMML is typically characterized by features such as splenomegaly, hepatomegaly, leukemic infiltrates in the lungs, and specific findings on peripheral blood smears. Children with NF1 and JMML may also have juvenile xanthogranulomas, but their occurrence does not appear to be higher than expected in other children with NF1. The frequency of lymphoreticular malignancies in adults with NF1 remains unclear.[40]

Hypotonia and impairments in coordination and fine motor function are frequent in NF1 children. The average IQ of NF1 people is 1 SD lower than general population and intellectual disability occurs in 5% of cases (frequency that is twice that in general population)[41,42]

Attention-deficit/hyperactivity disorder (ADHD) is present in 30%-50% of children and adolescents with NF1 and may persist into adulthood. 25% of children with NF1 meet standard diagnostic criteria for autism spectrum disorder (ASD). Psychiatric diseases such as mood disorders, anxiety disorders, and emerging personality disorders may also occur more often than expected among adults with NF1[43].

Seizures occur in approximately 5% of people with NF1, and their prevalence is slightly higher in adults compared to children. These seizures can be generalized, but more frequently, they are focal and often associated with a brain tumor, areas of infarction, or mesial temporal sclerosis. People with NF1 and epilepsy are more likely to have neurodevelopmental abnormalities. The approach to managing epilepsy in individuals with NF1 is similar to the approach used for those without NF1[44].

Hyperintense lesions, also referred to as unidentified bright objects (UBOs) or focal areas of high signal intensity (FASI), are observed in over 50% of children with NF1 when T2-weighted brain MRI scans are conducted. These lesions can appear in various brain regions, including the optic tracts, basal ganglia, brain stem, cerebellum, and cortex, and typically, they do not exert any mass effect. Notably, these typical UBOs do not show up on T1-weighted MRI images or CT scans. Pathologically, UBOs correspond to areas with spongiform myelinopathy[45]. They tend to reach their peak in terms of number and size at around the age of seven, after which they often regress. However, some may persist into adulthood. It's important to note that the presence of UBOs does not seem to be linked to the occurrence of seizures in children with NF1. Several studies have suggested that the presence, number, volume, location, or persistence of UBOs over time might be associated with learning disabilities or behavioral abnormalities in children with NF1[46,47]. However, findings in this regard have not been consistent across different research investigations and nowadays UBOs are more often to be consider a diagnostic criteria of NF1 rather than a pathological finding (fig 5).

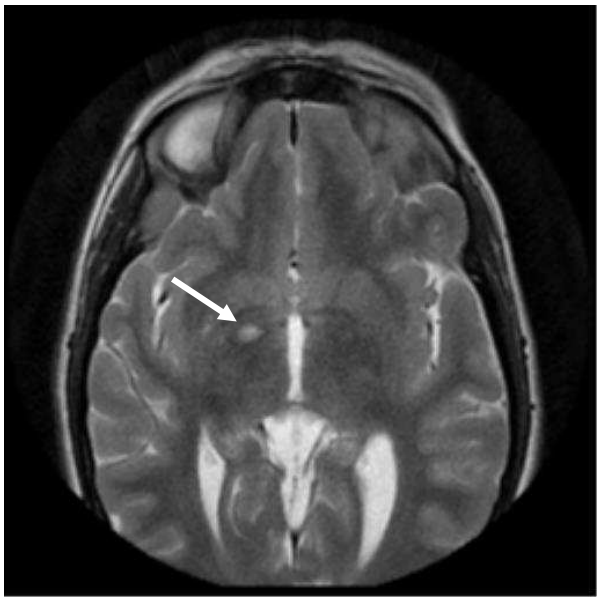

Fig.5 Axial T2-weighted MRI of a patient with NF1. White arrow indicates the UBO. Case courtesy of Henry Knipe, Radiopaedia.org, rID: 47703

Osseous dysplasia may occur as a primary abnormality in individuals with NF1 (typical of long-bone dysplasia), or in association with an adjacent plexiform neurofibroma or dural ectasia (vertebral or sphenoid wing dysplasia). Congenital pseudarthrosis of the tibia (CPT) is a rare condition with reported incidence of 1/60000 to 1/250000 live births but It is commonly associated with NF1. It is present in 2–6% of NF1 patients and has been suggested as a diagnostic criterion[48]. Nondystrophic scoliosis, which is not usually associated with vertebral abnormalities in individuals with NF1, resembles common adolescent scoliosis in its age at onset and more benign course. However in some cases treatment with growing rods in necessary[49]. Generalized osteopenia is more prevalent in individuals with NF1 than would typically be expected. Fractures also occur more frequently. Adults with NF1 tend to develop osteoporosis at a higher rate and at a younger age compared to the general population[50]. The exact causes of these bone changes are not fully understood, but individuals with NF1 often exhibit lower-than-expected levels of serum 25-hydroxy vitamin D, elevated serum parathyroid hormone, and signs of increased bone resorption. Both osteoblasts and osteoclasts, the cells responsible for bone formation and resorption, seem to function abnormally in the bones of people with NF1[51].

Vascular abnormalities in neurofibromatosis type 1 may arise anywhere in the cardiovascular system, and cerebrovascular involvement is the predominant feature of moyamoya syndrome (incidence of 2.3% in NF1)[52]. In people with NF1, strokes are more common and tend to happen at a younger age compared to the general population. Additionally, individuals with NF1 have a higher occurrence of

anatomically variant stenotic or ectatic cerebral arteries and intracranial aneurysms, with the internal carotid, middle cerebral, or anterior cerebral arteries being the most frequently affected[53].

Arterial hypertension affects 15%-20% of individuals with NF1. While it can develop at any age, it is more common in adults than in children. In many cases, no specific cause is identified, but hypertension may result from conditions like renal artery stenosis or mid-aortic syndrome, typically as a manifestation of NF1 vasculopathy especially in children[54].

Plexiform Neurofibromas and malignant peripheral nerve sheath tumors

Plexiform neurofibromas (PNs) are very common tumors in NF1, affecting approximately 50% of individuals with the condition. Histologically, they consist of numerous adjacent nerve bundles that are thickened and intertwined, immersed in a myxoid-like tissue with thick collagen fibers, giving them their characteristic multinodular appearance. Schwann cells and macrophages within the plexiform neurofibroma play a key role in producing numerous molecules (cytokines, chemokines, and growth factors), often with proinflammatory activity, which contribute to the growth of the neurofibroma itself[55]. The growth of plexiform neurofibromas is, therefore, multifactorial in origin. Plexiform neurofibromas can originate from any type of nervous structure, with the most commonly affected areas being the head and neck, back, and the upper and lower limbs. Deeper lesions, which stem from the roots of spinal nerves, often exhibit a "overflow" distribution, encircling the spinal column for a significant portion of its course, occupying the adjacent paravertebral spaces and altering its natural curvatures.

Plexiform neurofibromas are primarily congenital, and their size tends to increase during childhood. They typically reach their growth peak in the prepubertal phase and then, after the pubertal growth spurt, tend to stabilize. In essence, they grow along with the child. Although they are histologically benign and do not have the ability to metastasize, plexiform neurofibromas can lead to severe comorbidities such as pain, neurological deficits, restricted mobility, compression of internal organs (upper and lower respiratory tract, intestines, urinary tract), and blood vessels[56]. Furthermore, especially when they become particularly extensive, plexiform neurofibromas can transform into malignant peripheral nerve sheath tumors (MPNSTs). This possibility is observed in approximately 10% of cases[57].

MPNST are the most common malignant neoplasms associated with NF1. They tend to occur at a younger age and are linked to a worse prognosis in individuals with NF1 compared to the general population. Most, if not all, MPNSTs develop within preexisting diffuse or nodular plexiform neurofibromas. The primary clinical indicator of malignant transformation is persistent pain, which can be a new symptom or an exacerbation of existing pain. This pain may be accompanied by rapid tumor growth or changes in texture, which can be observed clinically or on MRI scans[58,59].

Genotype-Phenotype correlations

NF1 gene is located at chromosome 17q11.2 and spans approximately 350 kb and 60 exons. The first mutation in the *NF1* gene was identified in 1990[60]. Since then, hundreds of mutations have been reported, with over 80% of patients having a nonsense mutation, an insertion, or a deletion predicted to lead to a truncated protein product[61].

The 90–95% of NF1 causative variants are intragenic, while less than 10% are deletions involving the entire gene and flanking genomic regions[62]. To date, five genotype-phenotype correlations have been identified (fig 6). The growing number of genotype–phenotype associations is slowly but profoundly changing the clinical and genetic approach to NF1 patients. As genotype–phenotype correlations continue to increase, genotype-driven precision medicine could mark a major turning point in the management of NF1 by improving disease surveillance and patient stratification.[63]

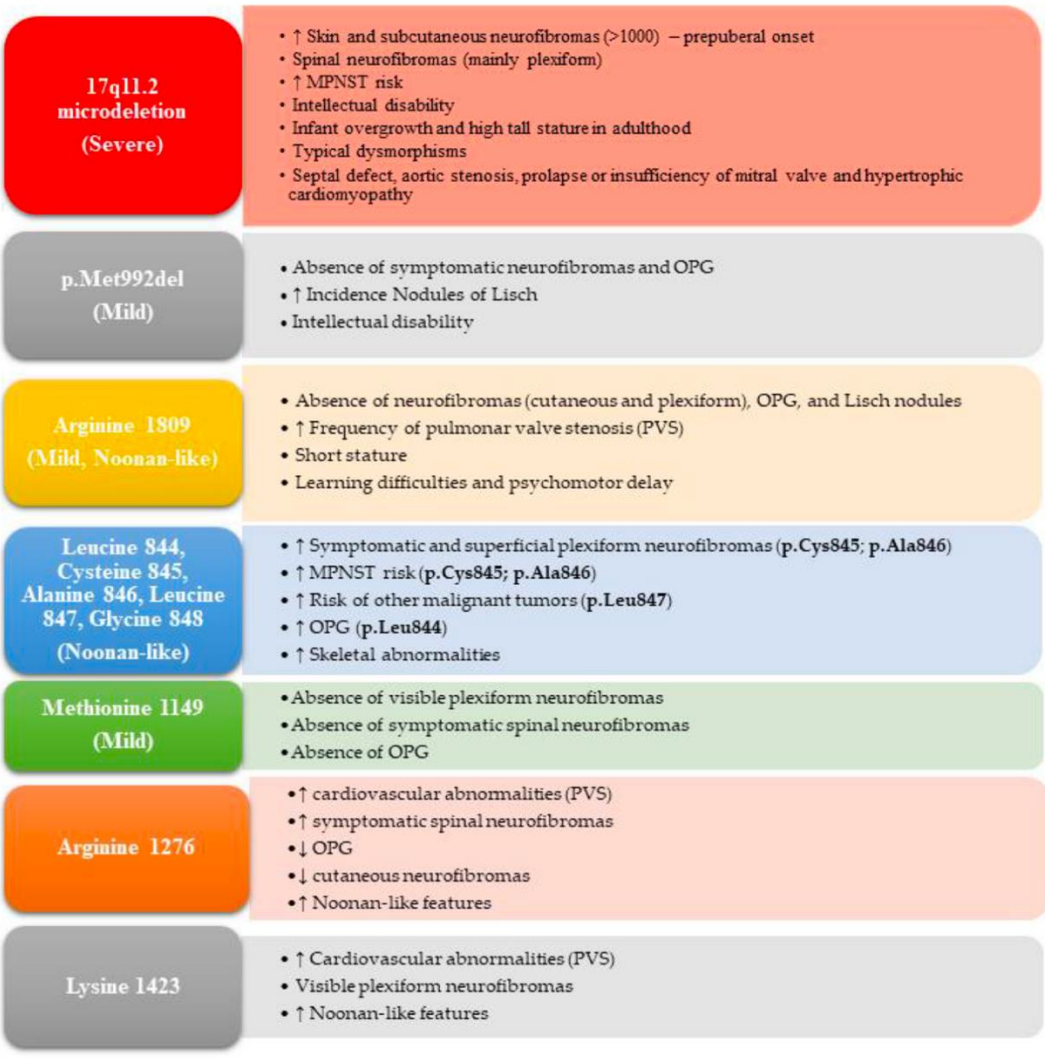

Fig.6 Genotype-phenotype correlation in NF1.[63]

Selumetinib

Selumetinib is a drug that belongs to a class of medications known as mitogen-activated protein kinase (MEK) inhibitors. It is primarily used in the treatment of certain types of cancer, particularly those that have genetic mutations affecting the MAPK/ERK pathway, which is involved in cell growth and division[64]. It has been the first molecule to demonstrate the ability of tackling the growth of PNs[65]. RAS pathway hyperactivation increases the production of transcription factors and causes cell survival and proliferation, leading to the typical features of NF1. The positive results obtained in the first study who described a clinical and radiological response to Selumetinib in 71% of their patients have been confirmed by following studies[66]. Selumetinib in NF1-PNs patients is administered at a dosage of 25 mg/m2, approximately 60% of the recommended dose as chemotherapy in adults. The information available on the drug's pharmacokinetics are mainly based on adults' data and few is known on the pediatric metabolism of Selumetinib[67,68]. At the same time, a target drug monitoring as a form of personalized therapy has never been attempted. Undoubtedly, it would be important to set up pharmacokinetic studies in order to determine absorption, distribution, metabolism, and excretion also in the pediatric population. Selumetinib in adults is known to be metabolized mainly in N-desmethyl selumetinib by CYP2C19 and CYP1A2, with some contribution from CYP2C9 and CYP2A6. In particular, CYP2C19 variants could influence Selumetinib pharmacokinetics and clinical response with major contributions of rs12248560, rs4244285, rs4986893 polymorphisms corresponding to CYP2C19*17, *2 e *3 alleles, respectively. While N-desmethyl selumetinib constitutes less than 10% of Selumetinib levels in human plasma, it is 3–5 times more potent than Selumetinib and contributes to approximately 21–35% of overall pharmacological activity making also its quantification[69].

On 31 July 2018, orphan designation (EU/3/18/2050) was granted by the European Commission to AstraZeneca AB, Sweden, for Selumetinib for the treatment of neurofibromatosis type 1. The medicinal product has been authorised in the EU as Koselugo since date 17 June 2021.

Transcriptome

The purpose of the holistic approach of omics sciences is to understand complex biological operating principles from an integrated perspective, where factors are studied together as pools of biological molecules that constitute a cell or a cellular or tissue system. The integration of omics sciences and technologies in the so-called complex systems biology improve the understanding of the system, considered as the collective of biological molecules that compose it. As an omics science, transcriptomics deals with the collective analysis of RNAs, taking into consideration the entire pool of cellular RNAs. Starting from DNA, transcribed into messenger RNA (mRNA), we proceed to proteins through the process of translation. Focusing on the first step, transcription, we examine the pool of intracellular RNAs. The transcriptome comprises all the RNA molecules present in a cell of a given tissue at a given moment. We can look at mRNA, which contains the genetic information encoding proteins, or at all the other forms of intracellular RNAs that mainly serve regulatory functions (from transfer RNAs or tRNA to ribosomal RNAs or rRNA, and even the perhaps less known micro-RNAs or long non-coding RNAs). We can say that the transcriptome represents a characteristic of the individual cell at a specific moment or condition. The expression of transcripts indeed changes depending on the conditions of the extracellular and intracellular environment. The subject of transcriptomics is precisely this: the cellular or tissue transcriptome, along with the variations it undergoes from cell to cell or tissue to tissue, following changes in the conditions in which the cell finds itself. Tissue-specific gene expression determines the morpho-functional phenotype of cellular and tissue types. In every differentiated cell and at any given moment in development, only a subset of genes is active. In all living organisms, the information contained in the genome is not expressed simultaneously but is finely regulated. Genes can be simplistically divided into three categories: constitutive expression (housekeeping genes), conditional expression (inducible or repressible), specialized genes (tissue-specific, stage-specific, which can in turn be constitutive or conditional). The activation or inactivation of gene expression in eukaryotes occurs based on cell differentiation during development, cell cycle regulation, and response to external mediators (hormones, growth factors, cytokines, etc.).To define a differentially expressed gene, it is considered whether its gene expression significantly deviates from the equal expression situation in the two states, for example, by comparing it to a threshold value to determine if it is over-expressed or under-expressed. Different transcriptomes simply represent different pools of activated and actively transcribed genes (which are then translated into proteins or remain as RNA with regulatory functions). Quantifying the transcriptome allows for understanding which genes are active in different phases of the cell cycle, development, or in response to specific signals from other cells and the

extracellular environment. From the one-gene approach, with omics sciences, we have transitioned to a large-scale approach[70]. In the former, the focus was solely on evaluating whether the gene of interest was expressed in a tissue at a given moment in development and how transcriptionally active it was. In the latter, the same parameters are considered, but multiple genes are taken into account simultaneously through the study of genome expression profiles or transcriptomes. Quantitative transcriptomics involves conducting a differential analysis of gene expression by comparing transcriptional profiles of two or more tissues or the same cell type under different conditions or at different moments (e.g., different developmental stages, cell cycle phases, health or disease). Transcriptome analysis is carried out using two different technologies: the first is based on hybridization (microarray), and the second is based on Next-Generation Sequencing (NGS) technologies. In 1995, the first microarrays based on cDNA molecule spotting were developed, and in 2002, high-density oligo microarrays were introduced. Since 2008, we have had the RNA-Seq technique available, which allows for messenger RNA sequencing through NGS techniques[71]. Nowadays NGS in strongly preferred due its key features: high Speed, high throughput, reduced costs and reduced human error.

BACKGROUND AND AIM OF THE STUDY

In the last decade, significant efforts have been made to identify drugs that could effectively reduce the growth of PNs, providing a viable alternative to surgery which is often unsatisfactory. The initial drugs used were interferon alpha-2beta and imatinib, but neither proved particularly effective. Since then, several drugs have been tested for the management of NF1, specifically for the treatment of PNs and MPNST.

The majority of drugs currently used to treat NF1 are repurposed agents originally designed for cancer treatment (fig 7). However, there are also ongoing efforts to develop new treatments based on unique aspects of NF1 pathology. High-throughput studies involving genomics, transcriptomics, proteomics, and epigenomics from both animal models and humans, in combination with molecular, cellular, and biochemical analyses of signaling pathways, have identified various potential targets for addressing NF1-related symptoms.

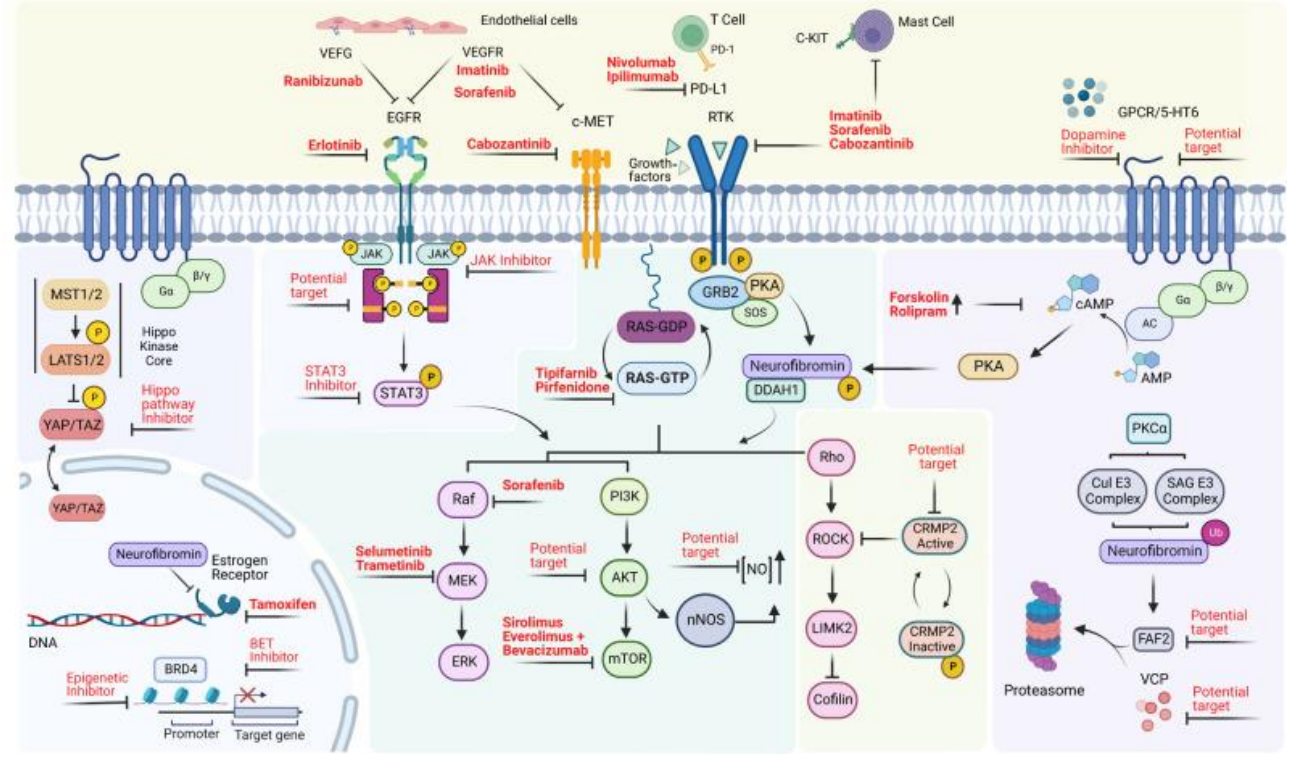

Fig. 7 The therapeutic potential of neurofibromin signaling pathways and binding partners[72]

In April 2020, selumetinib was approved by the US Food and Drug Administration (FDA) for treatment of 2-18 years old children with NF1-related symptomatic plexiform neurofibroma[66].

The basis on which a patient with NF1 and PNs might or might not respond to Selumetinib is rather unclear and the identification of a molecular blood signature may permit to obtain rapidly more information about possible responders and not responders and perform a tailored therapy avoiding adverse events that may worsen patients' quality of life. Much of the literature examined mostly the transition of PNs to MPNSTs and few studies investigated PNs gene expression profiles compared to normal cells. There is a lack of studies about gene expression patterns for NF1 in peripheral blood cells. To detect valuable biomarkers expressed in blood would be worthwhile given the easy accessibility of this tissue, using a minimally invasive technique (venous sampling).

MATERIALS AND METHOD

Inclusion criteria

All subjects with NF1 undergoing Selumetinib therapy, 2 years of age or older, were recruited. Additionally, those who had undergone at least one follow-up magnetic resonance imaging (MRI) after the initiation of the drug were included.

Study design and settings

Recruitment started on Marched 2021 at Rare and Metabolic Diseases Service in IRCCS Burlo Garofolo (Trieste). Before starting Selumetinib baseline medical history, clinical data and PN volume measurement by MRI has been recorded in a specific Excel database. Selumetinib was imported from the US and provided to all the subjects for compassionate use, since the drug has not still approved in Italy. Initial dose was 25 mg/m2 twice daily with careful attention to fasting. Every patients after starting the treatment with Selumetinib has been monitored, as clinical practice, with follow-up visits every 4-6 months. The visit included a thorough medical history with detailed discussion on the potential adverse events, a full clinical examination conducted by physicians with expertise in NF1, a complete ophthalmological exam, a pneumological visit with a spirometry (if allowed by age and compliance of the patient), a cardiological visit with electrocardiogram and echocardiogram, and laboratory blood tests (complete blood count, electrolytes, creatine, azotemia, aminotransferase, serum proteins, and creatinine kinase). Direct phone contact was introduced with the parents of the patients or the patients themselves. This allowed for immediate communication in the event of new symptoms, enabling them to promptly reach out to a physician. The physician would then record any potential adverse events and provide guidance on appropriate treatment or the discontinuation of the medication. MRI to assess the variation in size of the neurofibromas has been repeated 6-12 months after the beginning of the treatment (table 2).

	Months												
	0	3	6	9	12	15	18	21	24	27	30	33	36
Patient's enrollment													
Database generation, clinical data recording													
MRI data analysis													
RNAseq analysis													
Validation of genes resulting by RNAseq analysis													
Adverse Events reports													
Statistical analysis and correlation among clinical & laboratory data													

Table 2. GANTT Chart of the study

Plexiform Neurofibroma's MRI analysis

The PN volume measurement and 3D evaluation has been performed by a radiologist who works at Radiology service in IRCCS Burlo Garofolo (Trieste) with expertise in NF1 imaging evaluation. Volume assessment has been preferred to other variables (such as maximum diameters of the masses) due to the complex shapes and vast extension of most of the PNs. The volume assessment of plexiform neurofibromas was performed using Horos™ software, applying STIR or T2-weighted sequences from MRI examinations. The segmentation of plexiform neurofibromas, conducted on axial scans, was subsequently converted into a final volume, expressed in cm^3(fig. 8). Subsequently, MRI scans conducted prior to the initiation of Selumetinib treatment were compared to the latest available scans. The volume change has been expressed as a percentage. To facilitate comparison with previous cases reported in medical literature, the volume changes of PNs were categorized into three classes:

- "Tumor reduction" = mass shrinkage >20%
- "Tumor volume decrease <20%"
- " Tumor growth" = any expansion of the tumor progression

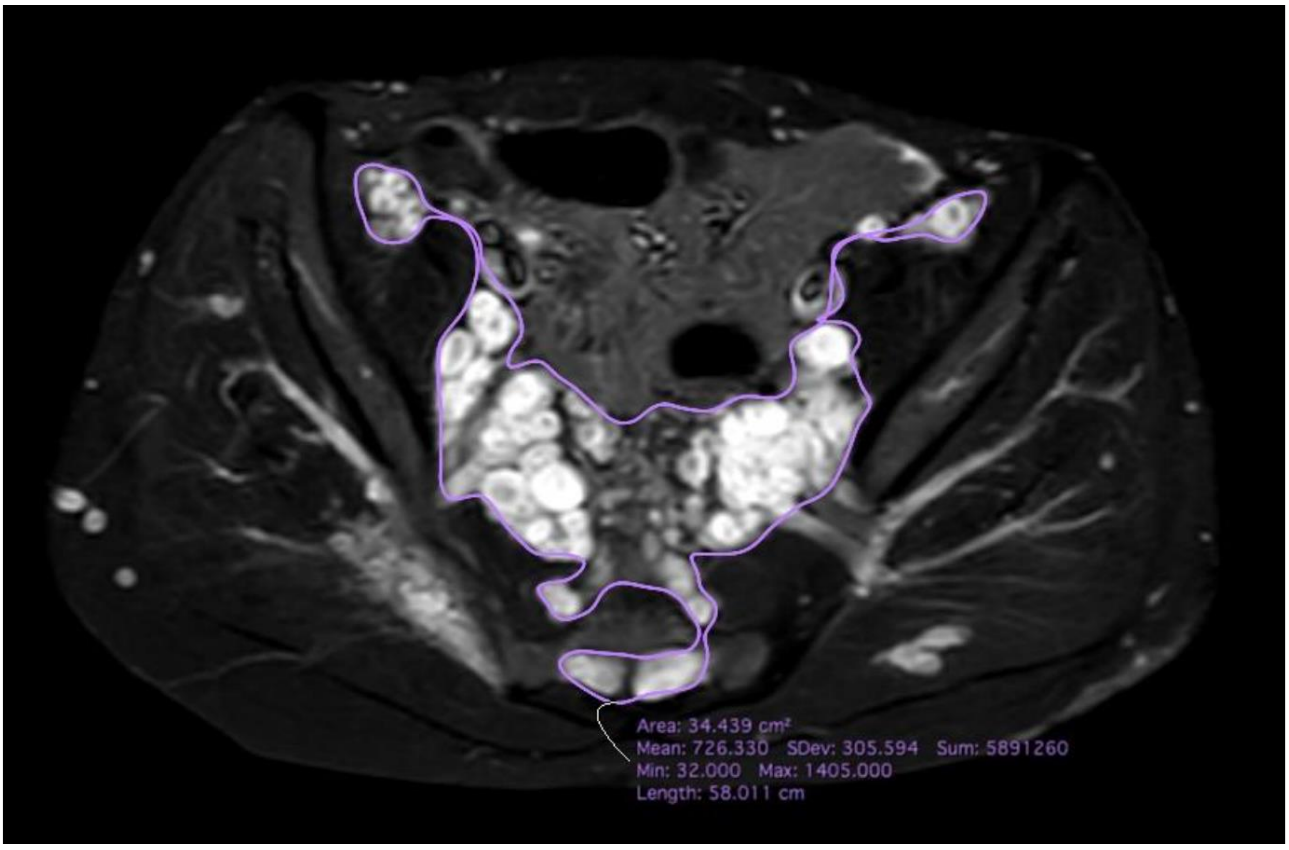

Fig. 8 Example of plexiform neurofibroma segmentation using Horos™ software

Transcriptome data

Transcriptomic analysis was performed on peripheral whole blood cells of the recruited patient by RNA sequencing. It has been compared to an equal numbers of healthy controls matched for age and sex. Blood samples to study RNA has been collected with PaxGene RNA tubes (PreAnalytix) at the first visit (before starting Selumetinib) and after 12 months. In patients who already started the treatment the RNA was collected at the first visit after March 2021 (month on which the study started). RNA isolation according to manufacturer's instructions of the kit (PreAnalytix). After samples quality controls RNA sequencing (RNAseq) has been constructed and analyzed by next generation sequencing (NGS). RNAseq raw data workflow has been conducted as follows: quality control by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), quality filtering by Trimgalore, read alignment to hg38 using annotation from GENECODE (https://www.gencodegenes.org/) with STAR aligner software, reads counting into genes by featureCounts package. Data has been normalized and analyzed for differentially expressed genes between distinct groups by DESeq2 (Bioconductor R package). Representative genes has been selected by adjusted p-value <0.05, according to the Benjamini-Hochberg method. Principal Component Analysis (PCA) has been used for representing in a simplified way the most macroscopic differences between samples. The complexity of the numerical gene expression matrix has been simplified into two dimensions principal components and express in a certain percentage of the variance. Gender differences were corrected before running PCA with the package limma in R environment.

Single gene comparison between groups was performed by the pairwise.t.test function in R environment considering normalized gene expression data (variance stabilizing transformations (VTS)).

Pathway analyses were performed on the most significant differentially expressed genes using according to the REACTOME pathway database via the function enrichPathway of the ReactomePA package.

RESULTS

Patients enrolled and demographic features

From march 2021 to march 2023, 14 Patients with NF1 were enrolled in the study (table 3). All recruited patients have an intragenic mutation in *NF1* gene. None of them have the same mutation and none have any of the variants described in the "genotype-phenotype correlation" section.
Every patients performed MRI to determine the PN volume before starting Selumetinib and after 12 months.
Among 14 patients analyzed, 11 were males (79%)(fig. 9). The mean age was 15 years. The mean age at which Selumetinib was started was 11,5 years old (fig. 10).

Demographic features	N (%), unless otherwise specified
Patients	14 (100%)
NF1 intragenic mutation	14 (100%)
Sex	
Male	11 (79%)
Mean age	15 years
Age at start of the treatment	
Mean	11,5 years
Range	3-19 years

Table 3 Demographic features of the patients

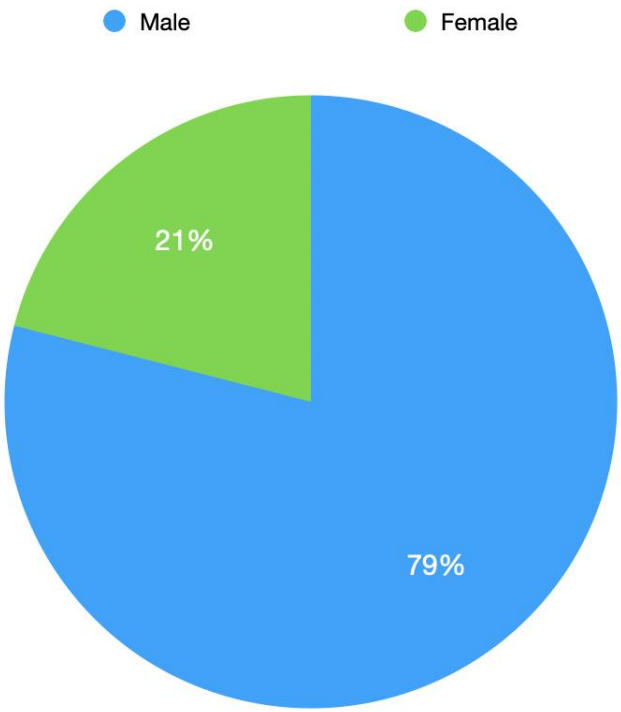

Fig 9. Sex distribution of recruited patients

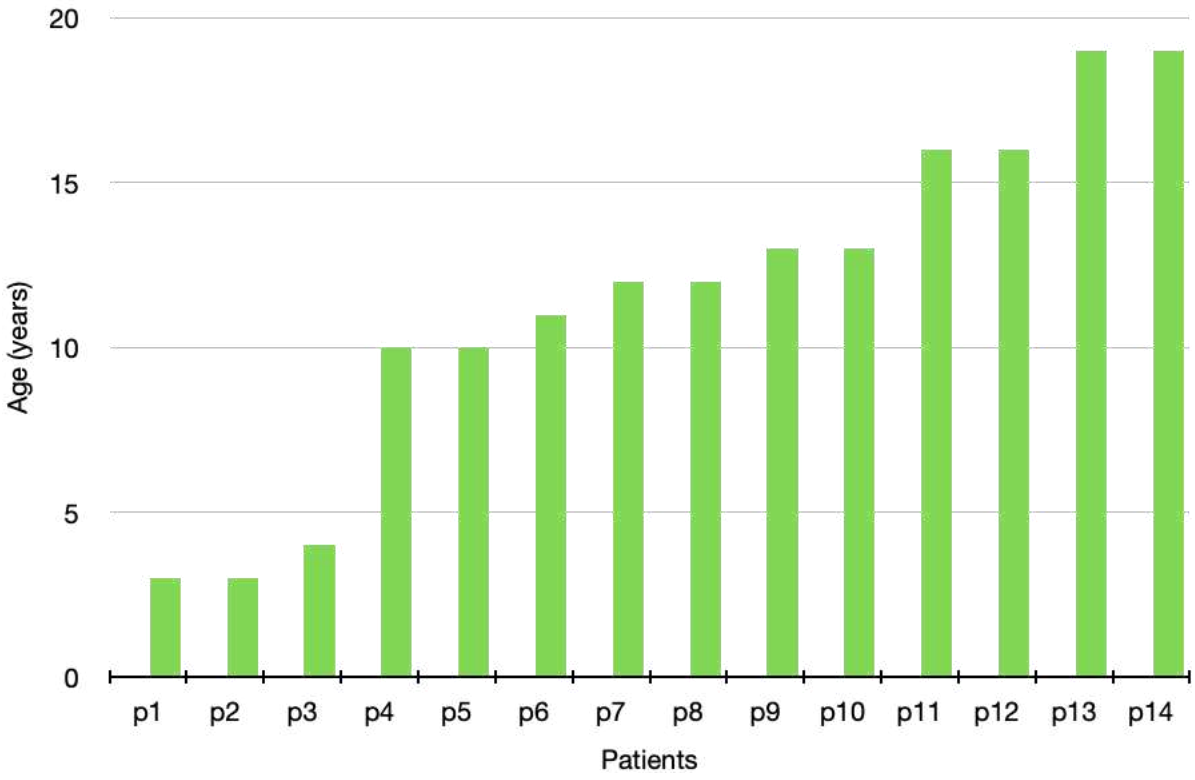

Fig 10. Patients‘recruited Selumetinib starting age

Plexiform Neurofibromas distribution and volumetric characteristics on MRI

The PNs distribution on the patients was: upper limbs (14%), lower limbs (22%), abdomen (14%), thorax (21%), head/neck (29%) (fig. 11). We observed a tumor response in all patients after 1 year of treatment (fig. 12). 5 patients (35%) had a tumor shrinkage more than 20% whereas 9 patients (65%) had a tumor volume decrease < 20% (fig. 13). No one increased the volume. The mean volume reduction was 22,9% (range 5,2% - 53,6%). Upon a more careful evaluation of the clinical data, emerged that p2 had been treated with Selumetinib not for a plexiform neurofibroma but for an OPG. This finding resulted in the inability to conduct the volumetric mass study and the transcriptomic analysis in p2 (fig.14)

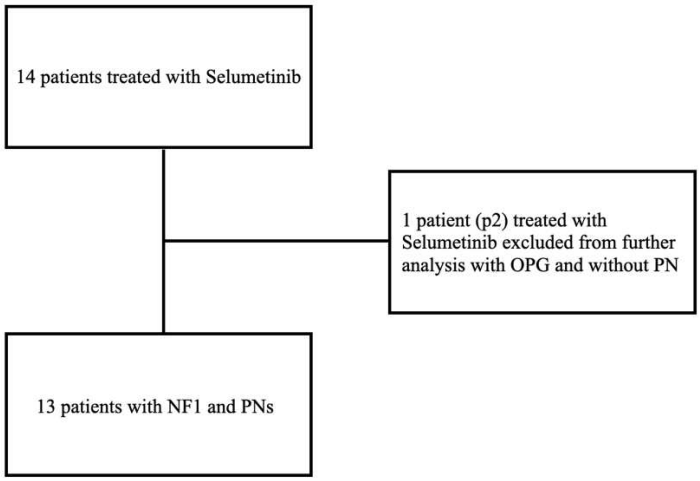

Fig. 14 Study analysis

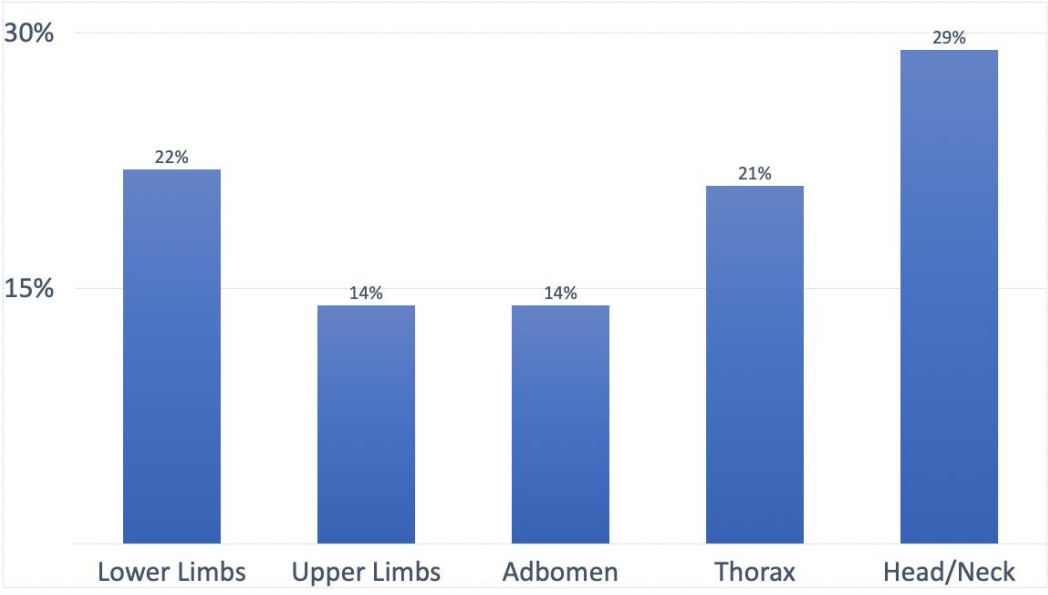

Fig. 11 PNs distribution among recruited patients

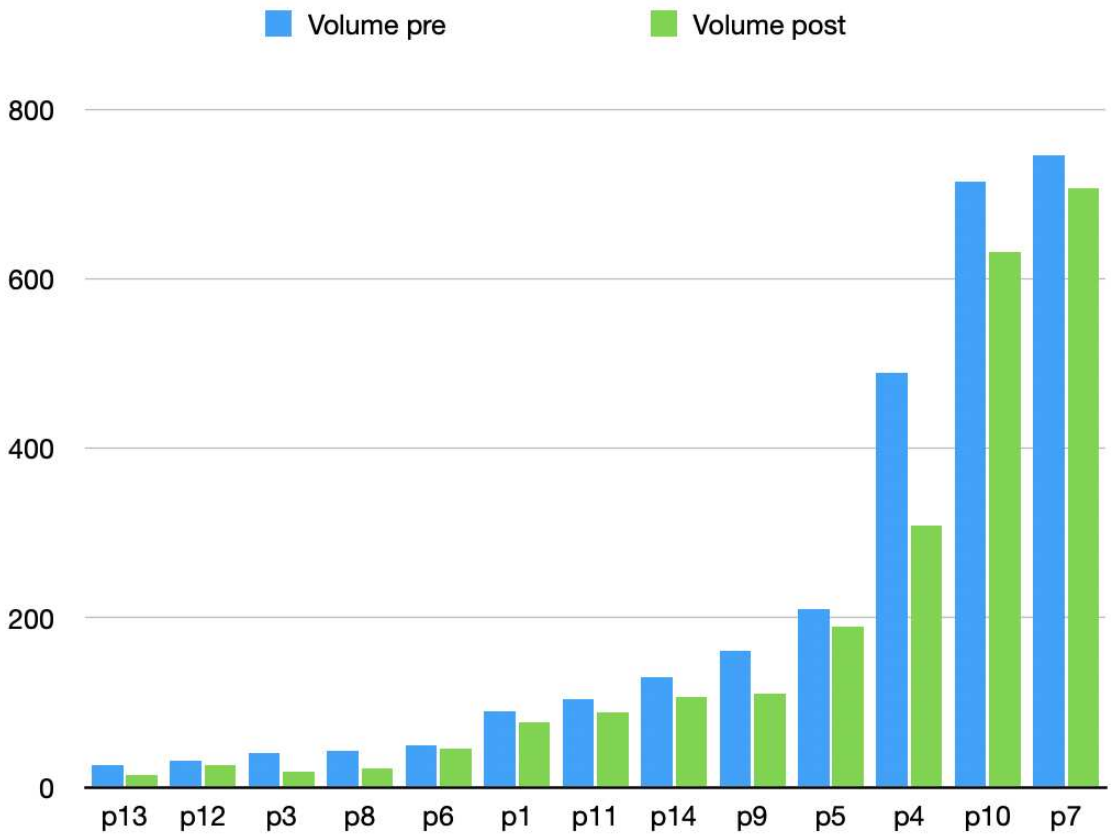

Fig. 12 PNs volume pre-Selumetinib (blue columns) and after 1 year of treatment (green columns)

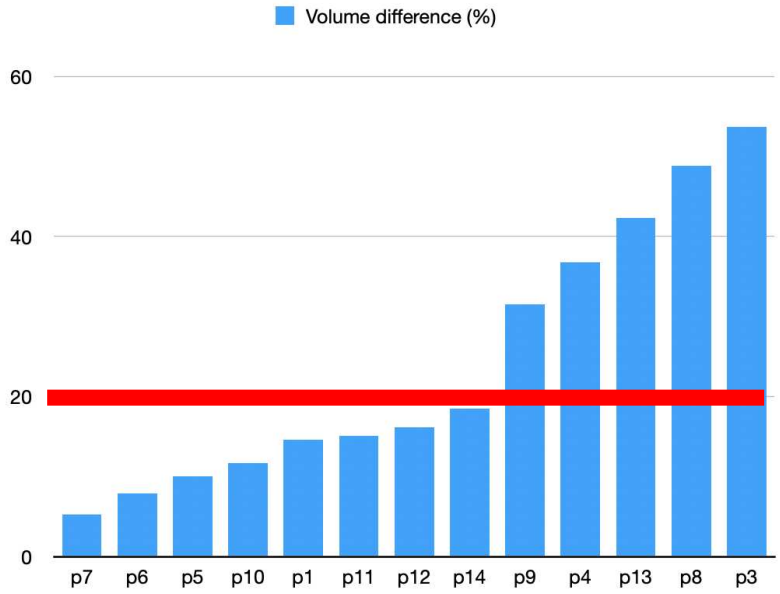

Fig. 13 PNs volume differences (%). The red line highlight the 20% chosen to define tumor volume reduction (< 20% under the red line; > 20% above the red line)

Side effects

Selumetinib has proven to be a well-tolerated drug because of none of the patients required discontinuation of the therapy. Almost all patients (93%) had one or more minor side-effects (fig. 15) mostly dermatological (86%), paronychia and abdominal pain/diarrhea. All side effects had resolved after appropriate treatment. Just in one case due to diarrhea and abdominal pain a temporarily Selumetinib dose reduction was necessary (from 25mg/kg/dose twice daily to 20mg/kg/dose twice daily). After 2 weeks the appropriate dosage was restored without side effects.

Acne only appeared in individuals aged 10 and above, while eczema had a broader distribution within the population, with a minimum age of 3 years and a maximum age of 19. Patients with acne used topical treatments in 77% of cases (drugs based on clindamycin and tretinoin) and oral therapies in 54% of cases (most frequently minocycline). No patient had such severe acne as to require oral isotretinoin. Eczematous lesions were treated based on severity and type, using topical steroid creams or emollients.

Another highly frequent event in our population was the development of paronychia, which occurred in 76% of patients. In five of these cases, onychectomy procedures were required. One patients needed surgical treatment on four separate occasions.

The onset of gastrointestinal symptoms (typically abdominal pain associated with loose stools or full-blown diarrhea) occurred in 62% of subjects, almost exclusively in the first weeks of treatment. Only occasionally it was necessary to prescribe symptomatic medications (especially loperamide) for patients to manage the symptoms at home.

Peripheral oedema of the lower limbs appeared in 6 subjects (29%). In one-third of the cases, localized cellulitis requiring oral antibiotic treatment subsequently developed.

In 5 subjects (24%), oral aphthous ulcers, asthenia, and irritability emerged. 3 subjects (14%) reported muscle cramps, and 2 (10%) experienced itching.

Excessive hair loss (*telogen effluvium*) of transient duration was observed in 3 cases (14%), and significant hair lightening with the appearance of blonde hair was noted in 3 cases (14%).

None of the treated subjects exhibited ocular, echocardiographic, electrocardiographic, or abnormalities at spirometry during serial ophthalmological, cardiological, and pneumological assessments.

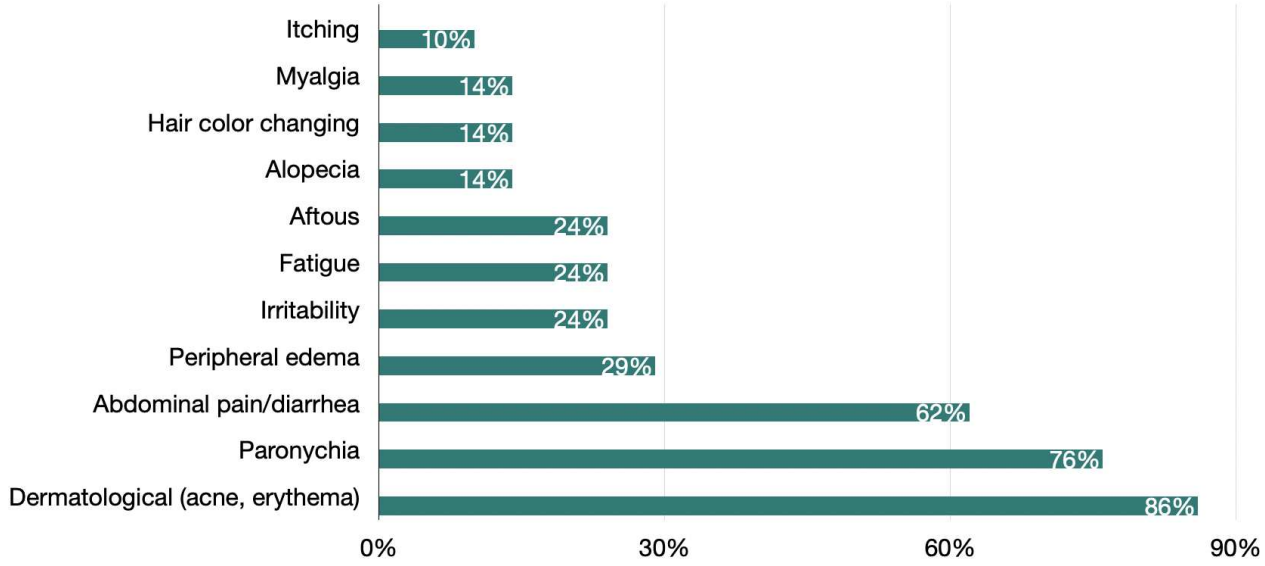

Fig. 15 Selumetinib side effects in recruited patients

Regarding alterations in blood tests, all subjects except one experienced an increase in Creatine Kinase (CK, average value 362 U/L, minimum 204 U/L, maximum 1126 U/L). No correlation was found between the CPK value and the occurrence of muscle-related symptoms. Less frequently, increases in ALT and AST were observed (43% and 38%, respectively), but never with significant values (average ALT 36 U/L, average AST 46 U/L). In contrast to CK, AST and ALT values were more prone to fluctuations, alternating between periods of mild positivity and others of clear negativity. CK values, on the other hand, remained consistently elevated in all subjects.

Transcriptomic data

RNA profile of 13 NF1-PNs patients after treatment with Selumetinib has been analyzed and the results have been compared with 8 healthy controls, matched for sex and age. 134 representative genes have been selected according to an adjusted p.value <0.05.

Figure 16 shows PCA which summarizes and visualizes the distribution of patients with NF1 after treatment with Selumetinib and healthy controls considering. The 500 most variable genes. Patients clustering together (patients enclosed in the circle) present similar gene expression profiles.

Due to clinical reasons and disease incidence not all patients have been enrolled perspectively and only for 4 subjects were collected RNA samples before and after starting the pharmacological treatment. RNAseq data of patients before treatment was used only to perform PCA.

Figure 17 shows the distribution according to the 500 most variable genes among the same patient with NF1-PN before and after Selumetinib treatment.

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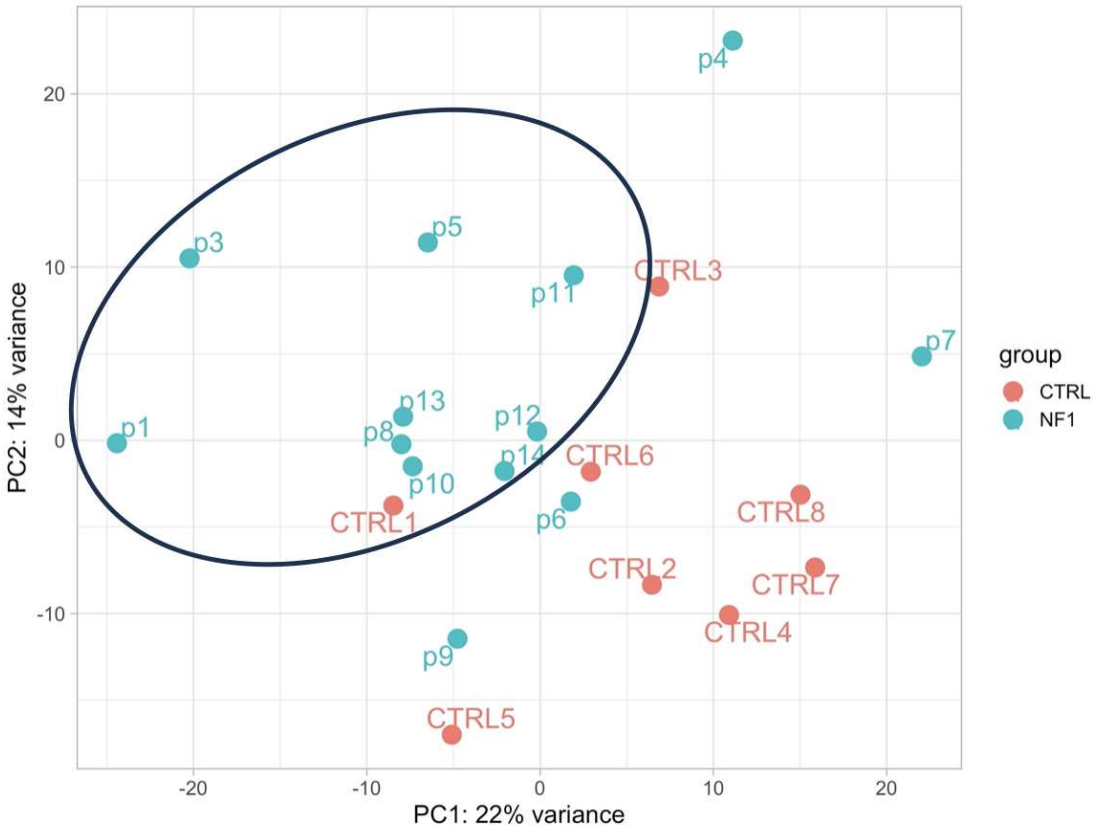

Fig. 16 PCA of patients with NF1-PN after treatment with Selumetinib (blue dots) and healthy controls (Orange dots). The black circle highlight a cluster of patients with similar gene expression.

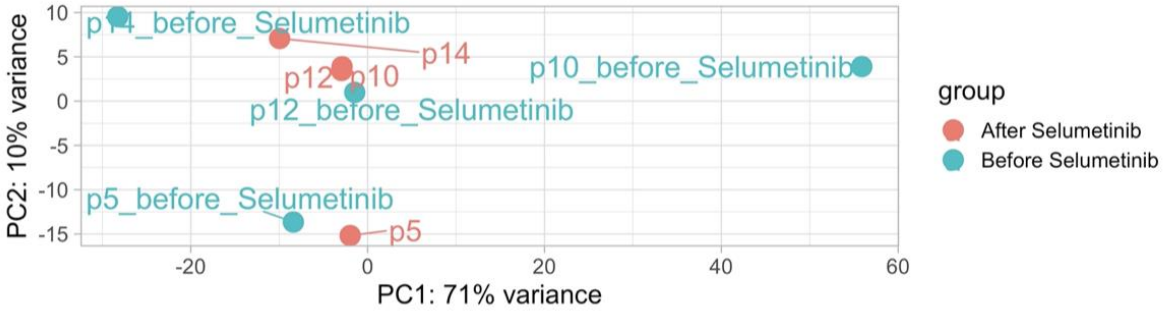

Fig. 17 PCA of patients with NF1-PN before (blue dots) and after the treatment (Orange dots) with Selumetinib.

Differential gene expression analysis was performed comparing patients with NF1-PN after treatment and healthy controls.

Genes have been chosen adjusted p-value < 0.05 according to Benjamin-Hochberg method. 134 genes were significantly differentially expressed (45 downregulated and 89 upregulated) (fig 18).

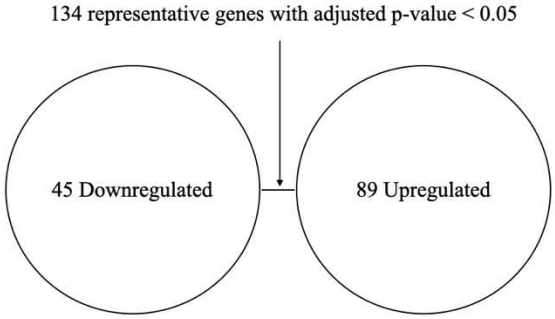

Fig. 18 Genes significantly differentially expressed

The biologically most relevant genes related to the subject matter molecular mechanism have been selected among the differentially expressed genes. The normalized expression of selected genes was compared across the three groups running a pairwise t-test.

Within the downregulated genes stand out *DUSP6* (p = 0.0038), *SPP1* (p = 0.012), *BPI* (p = 0.013), *MERTK* (p = 0.00022), *SIGLEC15* (p = 0.0027), *SIGLEC16* (p = 0.0033).

Figure 19 shows normalized gene expression values of the genes above across the three groups (patients after treatment with Selumetinib n=13, NF1 patients before treatment n=4, healthy controls n=8).

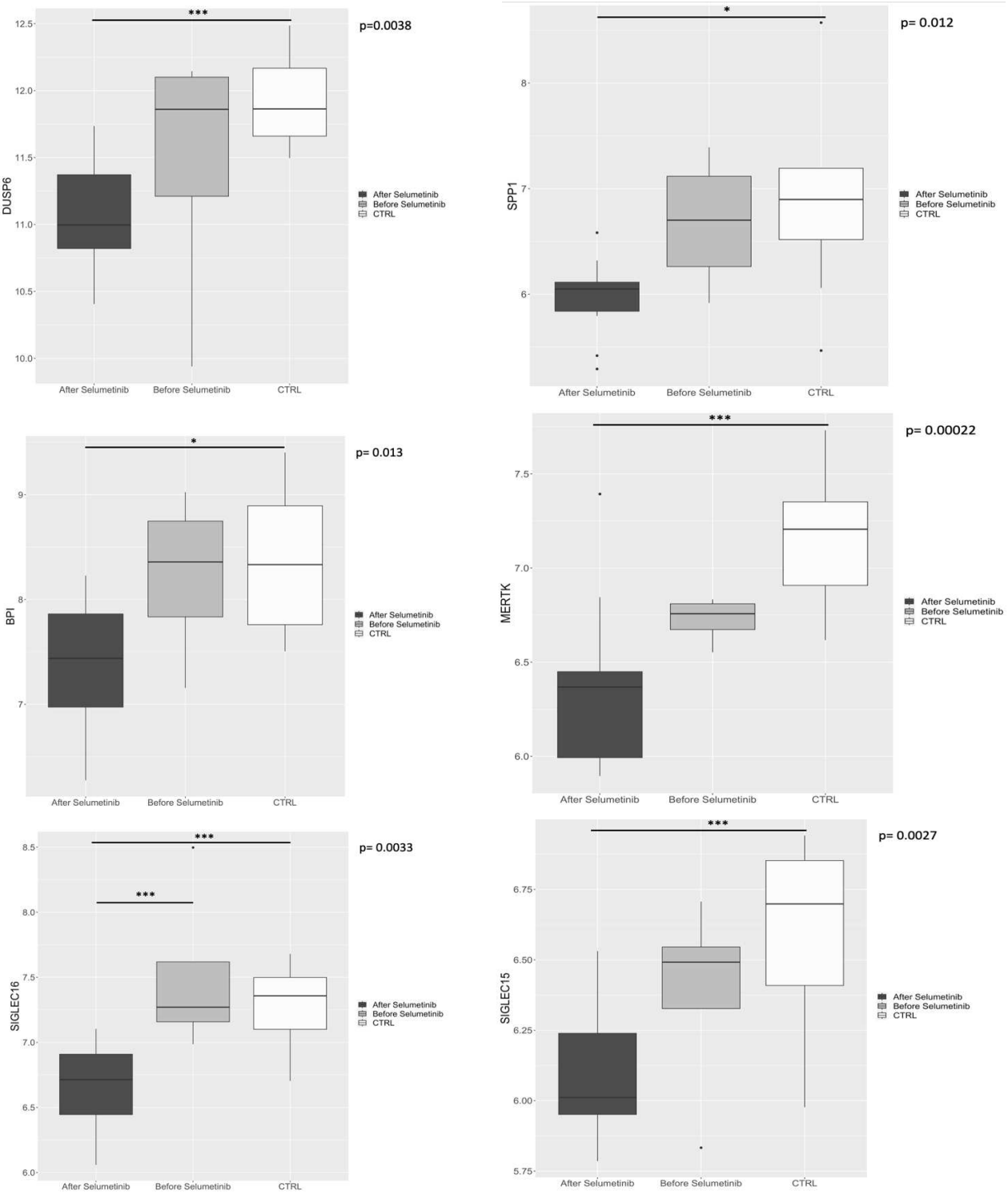

Fig. 19

Boxplot of normalized gene expression of most significant downregulated genes in patients before (grey) and after Selumetinib treatment (black) and healthy controls (white).

On the other side, 4 relevant biologically active genes were upregulated: *CD22* (p = 0.043), *BLK* (p = 0.0024), *BLNK* (p = 0.018), *CD79A* (p = 0.035) (fig 20).

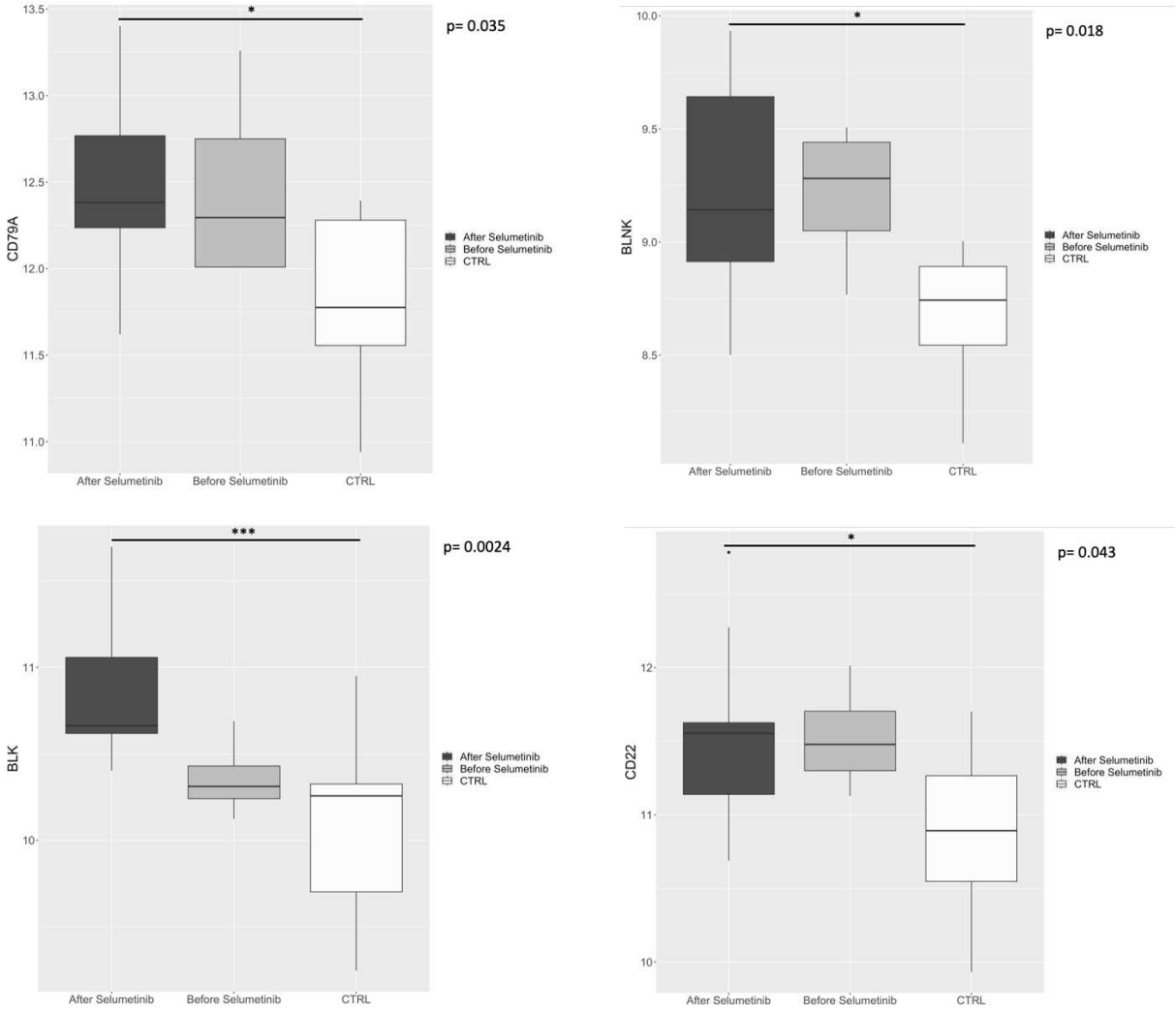

Fig. 20

Boxplot of normalized gene expression of the most biologically significant upregulated genes in patients before (grey) and after Selumetinib treatment (black) and healthy controls (white).

Pathway enrichment analysis of the statistically differentially expressed genes highlighted the "Antigen activates B Cell Receptor (BCR) leading to generation of second messengers" pathway including all the genes reported in Figure 20.

DISCUSSION

Being a doctor for a patient with NF1 with an inoperable, often disfiguring PN is quite challenging, especially when the patient is a child. This is because you don't know when or how the tumor will grow. These thoughts were shared by pediatricians and other specialists who used to care for patients with NF1, alongside the daily feelings of resignation and discouragement. Until recently, treatment options for NF1-PN were limited to complete resection (surgical removal of all affected tissue) or debulking (partial surgical removal). However, in many cases, surgical resection may not be possible due to location or size of the PN and is associated with a high risk of postoperative complications. Moreover, there is a high rate of regrowth after surgery, especially in children[73]. In the last decade, significant efforts have been made to identify drugs that could effectively reduce the growth of PN, providing a viable alternative to surgery. The initial drugs used were interferon alpha-2beta and imatinib, but neither proved particularly effective. A substantial reduction in tumor masses was achieved in only 5% and 14% of subjects, respectively, and both cohorts experienced major side effects[74,75]. The first promising results were seen with the clinical trial of Selumetinib[65], a drug already known in adult medicine and used in numerous oncology protocols. The rationale for its use lies in the fact that Selumetinib is an inhibitor of the activity of MEK1/2, key enzymes in the proliferative pathway initiated by RAS.

In this case series the clinical utility of Selumetinib has been confirmed. The drug has indeed proven capable of halting the growth of PN in all treated subjects. Additionally, the reduction in PN size was significant (below the pre-set 20% cut-off) in 35% of cases treated, with an average reduction of 22.5%. The natural history of PN predicts a growth rate inversely correlated with age: in pediatric age, therefore, we see the most significant tumor mass growth[76] with aesthetic and functional consequences that significantly worsen the quality of life. In previous studies[66] it was hypothesized that early initiation of the drug, and thus its use in children of particularly young age, could be associated with a better outcome. This data has not yet found further confirmation and will need additional investigation in the future, possibly in larger, shared cases. In our patients, no direct correlation emerged between the reduction in tumor mass and the age of starting Selumetinib, the location, or pre-treatment size of the PN. It should be emphasized that the clinical impact of growth arrest and partial reduction of tumor masses is, in some cases, outstanding (fig. 21).

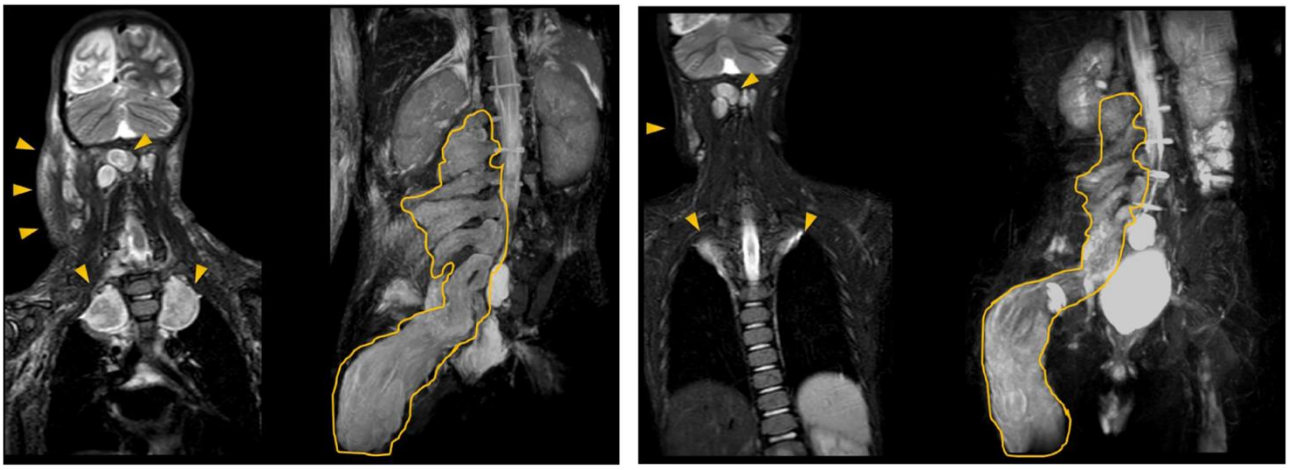

Fig. 21 MRI coronal STIR sequences of the PNs of the neck, thorax, dorsum, and right thigh in a NF1-PNs patient before (left side) and after (right side) 1 year of Selumetinib

In terms of Selumetinib's safety profile, this study confirms that it is generally well-tolerated. None of the patients had to discontinue treatment due to the severity of side effects, which were almost always of mild or moderate. In comparison to specific NF1-selumetinib case studies[65,66], we observed a higher incidence of dermatological issues (86%) and paronychia (76%) in our population. However, these percentages of dermatological effects are in line with previous studies[77] where Selumetinib was used as a second-line chemotherapy in the adult population.

In this study, we pursued to find a correlation between the drug's efficacy and transcriptomic data. One patient (p2) undergoing treatment with Selumetinib was excluded from the mRNA analysis because of he did not have any PNs but rather an OPG, and he started the compassionate treatment as a second-line chemotherapy. Through statistical analysis of the data, comparing patients treated with Selumetinib to healthy controls and NF1-PN patients before and after the treatment, intriguing data emerged. Among the differentially expressed genes, we selected a gene subgroup according to the pathways they are involved in. It became evident that there is a downregulation in certain genes indirectly related to the MEK pathway, as we expected. The first example is the *DUSP6* gene. Until a few years ago, it was believed that this gene, a cytosolic dual specificity phosphatase (*DUSP6*)[78] functioned as tumor suppressors. Thus, decreased expression of *DUSP6* gene occur in human cancer[79,80]. But recently, it has been demonstrated that *DUSP6* is upregulated in certain types of tumors[81,82], and it is believed that in these cases, it may help the tumor itself in adapting to excessively high levels of growth factor signals. Moreover a recent study confirmed that *DUSP6* expression is important for growth and survival in NF1-deleted MPNST and suggest that targeting DUSPs may be

a therapeutic option for treating MPNST [83]. In animal model of NF1-treated with Selumetinib *DUSP6* was downregulated[84] and our study confirmed this findings in humans.

The expression of *SPP1* (and its protein product osteopontin) is regulated by Wnt signalling, one of the pathways that has been identified as playing a major role in the malignant progression of PNs to MPNSTs[85,86]. Our study is the first to demonstrate the downregulation of *SPP1* espression throught Selumetinib treatment. As a consequence of this, it is hypothesized that the drug may reduce the likelihood of the PN transforming into MPNSTs.

The *MERTK* gene (also known as MerTK or c-Mer) encodes a receptor called Mer tyrosine kinase. The *MERTK* receptor is involved in various cellular processes, including those related to phagocytosis of apoptotic cells and the regulation of the immune system. *MERTK* is part of the Tyro3, Axl, and MerTK (*TAM*) receptor family and has been associated with various aspects of cell biology, including cell signaling and cell growth control[87]. In our findings, it is highlighted that *MERTK* appears to be downregulated even prior to Selumetinib treatment. Probably, with MEK inhibition, the production of this receptor, which serves as an activator of the pathway, decreases. Further studies are necessary to confirm this findings.

SIGLEC15 was initially identified as being highly expressed in giant cell tumors of the bone and was subsequently revealed to play a role in regulating osteoclast differentiation and bone remodeling[88]. Recent research has shown that *Siglecs* is also broadly upregulated in the tumor area[89,90] and in tumor-infiltrating myeloid cells in humans. *Siglecs* are considered a novel anti-tumor target as they have the capability to consistently suppress T-cell responses and induce immune evasion within the tumor microenvironment[91,92]. There is no evidence that *SIGLEC15* or *16* are associated with NF1-PNs patients. In our case series, gene expression significantly decreased, likely due to the downregulatory effect of Selumetinib.

Bactericidal/permeability increasing protein (BPI) is a major constituent of neutrophils (0.5 to 1% of total protein) and in smaller amounts also present in eosinophils[93]. It has antibacterial activity against the Gram-negative bacterium and is present in the skin. Comparative analysis of *BPI* expression reveals a significant downregulation in patients treated with Selumetinib. While not reported in the literature, this could potentially account for the nearly complete presence of dermatological side effects in treated patients.

The transcriptomic analysis highlighted the upregulation of specific genes: *CD79A, BLK, BLNK*, and *CD22* are all related to B cells of the immune system. As can be seen from fig. 19 there is a significant difference in genes' expression between patients with NF1 (before or after Selumetinib treatment) and healthy controls. The *CD79A* gene codes for a component of the CD79A/CD79B protein, which is part of the B-cell receptor complex. This receptor is essential for B-cell signaling and activation.

The *BLK* gene codes for a kinase protein involved in B-cell signaling. *BLK* plays a crucial role in regulating B-cell activation and antibody production. *BLNK* is involved in B-cell signaling and acts as a bridge between B-cell receptors and intracellular signaling pathways, helping to transmit initial signals to intracellular pathways[94]. The *CD22* gene codes for a cell surface protein expressed on B cells. *CD22* is involved in regulating B-cell activation and inhibiting excessive activation, helping to maintain immune system homeostasis. In a recent study clinical and immunophenotypic data of a cohort of patients with RASopathies and mTORopathies were collected. Among the studied lymphocyte subsets, the only consistent alteration regarded an increased percentage of immature B cells (recent bone marrow emigrants) and an increased percentage of double negative T cells. Borderline immune abnormalities were present in a significant proportion of subjects and adenotonsillectomy was performed more frequently than expected for the general population, no major immune disturbance was found in this cohort of patients[95]. Further studies are necessaries to better understand this immune dysregulation.

This is a monocentric study and has several limitations. First the small number of patients recruited and the absence of before and after Selumetinib transcriptomic data for all patients.

Genetic analysis were performed directly on *NF1* gene guided by an evocative clinical picture, therefore we can not exclude the presence of other mutations which might affect gene expression of analyzed pathways.

Nevertheless, the transcriptomic data is highly interesting and could serve as a basis for the development of valuable biomarkers to assess the drug's efficacy or even to monitor the potential malignant transformation of a PNs into an MPNSTs. Further studies are crucial for advancing precision treatment in NF1.

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