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Neurofibromatosis type 2: results of an Italian multi-center study on the clinical features of the disease in childhood and on the response to treatment with bevacizumab

Andrea D. Praticò

Tutor: Professor Salvatore Salomone
Co-tutor: Professor Martino Ruggieri

Index

1st Chapter

The syndrome

1. Neurocutaneous Disorders

Neurocutaneous disorders are multisystem diseases mainly affecting the skin and the nervous system, as well as other organs (1, 2). The concurrent involvement of nervous and cutaneous cells is due to their common ectodermal origin since the very early stages of embryonic life (3). Ectoderm is the more external of the three primary germ layers, which include also mesoderm (intermediate layer) and endoderm (internal layer) (4). Each of these layers forms different tissues in the very intensive process of cell differentiation that is the embryonic period (i.e. the first seven weeks of gestational age); in particular ectoderm differentiates very early in external ectoderm (forming the skin and the external layer of the glands, tooth enamel, hair, nails, lens and cristallinum in the eye) and the neuroectoderm. This last generates the neural crest and the neural tube. Neural crest is the progenitor of several cellular types including odontoblasts, dental papillae, chondrocranium, tracheal and laryngeal cartilage, connective tissue of head and neck glands (pituitary, salivary, lachrymal, thymus, thyroid) as well as dermis, adipose tissue, some endocrine cells (chromaffin cells of the adrenal medulla) and components of the peripheral nervous system, including sensory neurons and glia of the dorsal root ganglia, cephalic ganglia (VII and in part, V, IX, and X), satellite glial cells of all autonomic and sensory ganglia, Schwann cells of all peripheral nerves. Melanocytes are thought to derive directly from this part of the neuroectoderm.

The neural tube gives origin to the whole central nervous system, from brain to spine.

The exclusive involvement of cells derived from the ectoderm, more precisely an uncontrolled proliferation and/or maturation of neuroectoderm components, is the main cause of the neurocutaneous disorders (3, 5).

In spite of their "common" neuroectodermal origin, neurocutaneous disorders are extremely heterogeneous diseases, with a high variability of clinical manifestations and phenotypic presentations among the different disorders and within individuals affected by the same syndrome. The most common, **Neurofibromatosis type 1** (NF1), inherited by an autosomal dominant fashion, presents with café-au-lait spots in the skin, cutaneous neurofibromas, nodules in the iris (Lysch nodules), (mostly) benign brain tumours, all with an extremely heterogeneous presentation (6); other common disorders are **Tuberous Sclerosis Complex,** characterized by brain tumours (mainly giant-cell astrocytomas), hypomelanotic skin macules, lung and kidney involvement (7), **Sturge-Weber Syndrome** a mosaic neurocutaneous disorder presenting with capillary malformation in the skin and in the brain (8), **Von-Hippel Lindau Syndrome,** that presents with benign and malignant tumours of the central nervous system, kidneys, adrenal glands, pancreas, and reproductive organs, as well as hemangioblastomas of the brain, spinal cord, and retina (9), and **Neurofibromatosis type 2** (NF2), which shows an involvement of central nervous system with schwannomas of the eight cranial nerve (i.e. auditory-

vestibulary nerve) and other cranial nerves, meningiomas and spinal tumours, as well as involvement of skin with cafè-au-lait spots, plaques, cutaneous tumours (10). A third form of neurofibromatosis, "**Schwannomatosis**", characterized by the onset of multiple schwannomas in the peripheral nervous system, has been only recently classified as a separate disorder, being for decades considered as a "peripheral" form of NF2 (10, 11).

2. Neurofibromatosis type 2

2.1 Generalities

NF2 is a monogenic autosomal dominant disease caused by mutations of the *NF2* tumour-suppressor gene. Affected individuals are predisposed to develop central nervous system tumours, usually (bilateral) vestibular schwannomas, meningiomas, schwannomas of other cranial nerves, spinal roots and peripheral nerves, ependymomas, focal amyotrophy, peripheral neuropathy and eye cataract (12).

Even before different genetic causes of NF1 and NF2 were discovered in the second half of XX century (13-15), doctors and caregivers had considered NF2 as a severe disorder, with a high mortality due to the aggressiveness of the brain tumours, thus differentiating the syndrome from NF1, a severe, but in most of the cases a non-life threatening condition.

The high mortality of NF2 patients, even at younger ages, has justified in the modern medicine aggressive treatments involving high-dosed

antineoplastic drugs, radiation and neurosurgery, all associated to severe adverse effects such as deafness, blindness, loss of equilibrium, inability to walk, and other neuro-disabilities. Only in the last decade, with the introduction of newer, more selective, anti-neoplastic drugs, a targeted treatment has been carried out. The rarity of the disorder and the impossibility to test such protocols in large cohort of patients (NF2 has an incidence of 1:25,000) has been sometime by-passed by larger experimental procedure, with selection of patients through entire nations or group of nations, in order to provide evidences of the beneficial effects (or the failure) of these drugs in the treatment of the syndrome. In the last ten years Bevacizumab, a super-selective Vascular Endothelial Growth Factor inhibitor, has showed promising results in terms of tumour regression and improvement of deafness, vision, headache.

The results of a multi-centre study aimed to characterize the phenotype at onset and in childhood of Italian NF2 patients, together with a clinical trial on bevacizumab use for NF2-related tumours are herein reported.

2.2 History of Neurofibromatosis in Human Medicine

The history of neurofibromatoses, in particular the more frequent and common NF1, can be traced to ancient times, if descriptions of grotesque or distorted persons are considered (16, 17). By contrast, reports of early examples of *neurofibromatosis type 2* (**NF2**) or *schwannomatosis* sufferers can be traced in descriptions, illustrations or portraits dated as back as the 18th century(18).

For neurofibromatosis type 1, the earliest examples of people presenting (skin) nodules resembling *neurofibromas* (or *plexiform neurofibromas*) can be found in Ancient Egypt (19), in the coinage of the Parthians kings [247 B.C.](20), and later, in medieval and modern ages, in manuscripts (21) and drawings (22), as well as in descriptions of "grotesque" or distorted individuals (at that time called "monsters") (21, 23). Detailed clinical descriptions were made at the end of the sixteenth century, with Ulisse Aldrovandi, credited as being the first to report, in 1592, a "full clinical case" of a short man with a large tumour resembling an isolated plexiform neurofibroma (24). In the following centuries, men probably affected by NF1 were portrayed with an increasing number of details(25, 26), and, during the Enlightenment, aside of drawings, sculptures and other artistic representation, the first clinical reports in English language were published, by Akenside in 1768 (17), and by the Irish surgeon Robert William Smith in 1849(27). The historical name of the syndrome came after Baron Friedriech Daniel von Recklinghausen, who in 1882 was the first to confirm the "neurologic" origin of skin tumours and to name them neurofibromas(28).

2.3 Neurofibromatosis type 2: from a "variant" to a distinct disease

NF1 cutaneous manifestations are often evident especially in the peripheral tissues, and for this reason, in the previous centuries, it was considered as the "peripheral neurofibromatosis". By contrast, NF2 has fewer cutaneous

manifestations, while, in most of the cases, central nervous system tumours occur. For this reason, the disease has been considered as “central neurofibromatosis” for almost two centuries, and only in the last 40 years recognized as a distinct nosographic entity. (18).

As tumours of the VIII cranial nerve (acoustic nerve) arise almost always in NF2 patients, the first patients affected by this disease can be traced with the first descriptions of these tumours, which date back to the half of the XVIII century, when Eduard Sandifort, from the University of Leiden (Netherlands), described such a neoplasm in an autopsy (29). After 40 years, such tumours were termed as “neuroma”, because of the presence of nervous cells, by Louis Older (18).

Only in 1822 a living patient affected by bilateral acoustic neuromas was described, by the Scottish surgeon John H. Wishart, who presented his case to the Royal College of Surgeons in Edinburgh: he was a 21-year old baker, who had presented a blind eye since his infancy and during his childhood fell constantly. After that, he became deaf in both ears, blind and affected by intractable headaches, vomiting and paroxysm of pain and facial twitching. For this reason he was “thoughtfully” operated by the Surgeon, dying a few days later because of an infection (30). At the post-mortem examination, Wishart observed numerous tumours, some of which located in the dura mater (i.e., meningiomas) while others arisen from the cranial nerves (i.e., cranial nerve schwannomas). Wishart described lesions arising from the

5th and 11th nerves as well as from both auditory meati (i.e., schwannomas). The last of these, in retrospect, were almost certainly acoustic neuromas. Importantly, Wishart associated the acustic (cranial) nerve tumours with the occurrence of meningiomas, which at that age were already considered two distinct type of tumours arising from different cell types (nerve cells, meninx).

After this description, this aggressive version of the disease affecting young patients has been denominated "***Wishart subtype***" or "***Wishart's variety***" for NF2 with an early and aggressive clinical course [i.e., *childhood onset NF2*](31, 32).

Other cases in that years were reported by French pathologist Jean Cruveilhier (1835), who analysed the intracranial effects of acoustic neuromas (33), and by Knoblauch in 1843, who distinguished, using only a few instruments, "morbid process" effects to "original vice of conformation" in the biopsy of human brains (i.e. inflammatory vs. cancerous tissues) (34).

In the following years, better optics for the microscopes and improved staining techniques, allowed scientists to examine sections of tumours and tissues, and in the second part of the XIX century, Virchow was able to distinguish "malignant tumours" (capable to metastasize and with rapid growth) from "benign tumours" (who presented as histologically similar to the tissues of origin). Based on this similarity, they grouped tumours in fibrous, bony, fatty, neural or vascular (35). In the same years, the reports from Robert William Smith, on patients affected by "general development of neuromatous tumours"

(and not more isolated tumours) were probably the first scientific descriptions of neurofibromatosis (many of them probably NF1, and thirty years before Von Recklinghausen)(36). One of his patients could represent a first reported case of NF2 or Schwannomatosis (37): he was a 35-year-old man who presented a large tumour on the right side of the neck thought to be malignant, and a second, sublingual, tumour. He was not operated, readmitted later in an emaciated state and died towards the end of the year "with hepatic symptoms". He had developed another "giant" tumour in his left tight and several hundreds of other dimensions, thought to be "neuromata". At microscopy, they presented a fibro-cellular structure, with oval or elongated nuclei and there was no trace of malignancy (i.e. "nerve-tubes" in the specimen). What could have affected the patient, given also his emaciated state, was probably a malignant degeneration of neurofibromas (malignant peripheral nerve sheath tumour), a rare, but lethal, complication of NF1, which usually present as large, globoid and encapsulated tumour; however, the results of the biopsy contrast this hypothesis. The drawing of the tumours of the neck, present in Smith's book, give an impression of a large, globoid, encapsulated, eccentric lesion, as usually appears in schwannomas, which also lacks "neurites" (what they reported as the absence of nerve-tube). In this case, this patient would be the first literature description of a NF2, which may present with large schwannomas in the peripheral nerve. The small, slightly raised, rounded lesions present in the skin of the trunk of the patient could represent cutaneous schwannomas (i.e. NF2-

plaques), another NF2 hallmark or (b) schwannomatosis, this last being a recently recognized neurocutaneous disease characterized by multiple peripheral schwannomas (38).

Thirty years later, in 1882, von Recklinghausen published his landmark monograph on the disease that would later become known as NF1 or Von-Recklinghausen disease (39), and in 1897, Mossé and Cavalié first used the term *central neurofibromatosis* to denote a variant lacking the peripheral manifestations, recognizing also that bilateral acoustic neuromas may be accompanied by numerous other cranial nerves neuromas (40). In the same years, based on advanced histological procedures, Stenberg recognized that the nervous tumours of "central neurofibromatosis" derived from Schwann cells and not from the neurons. These results were then confirmed by the Uruguayan pathologist Jose Juan Verocay, in 1910, which coined for these tumours the term "neurinoma" (meaning *nerve fiber*s tumour), in contrast to von Recklinghausen's "neurofibroma" (18).

After a few years, the distinction between central (NF2) and peripheral (NF1) was clinically accepted, given the increasing number of descriptions of patients affected by the rarer central form: these individuals were reported as having fewer skin manifestations but almost constantly bilateral acoustic tumours, multiple neurofibromas in peripheral nerves, extradural and intradural spinal nerves (41).

In the same year, Fraenkel and Hunt, at Cornell University in New York, described a patient with bilateral acoustic neuromas, and fewer other cutaneous signs: they highlighted that, despite a common origin with other (peripheral) neurofibromas and fibromas tumours reported in other patients, "central" forms of neurofibromatosis had to be distinguished from the "neurofibromatosis of the skin, cerebrospinal and sympathetic nerves" (42).

Notably, in the same period, Biggs, reporting the post-mortem examination of a patient with bilateral acoustic neuromas, noted that "the largest acoustic tumour was on the side of a "milder" deafness being the first to report the clinical vs. anatomical discrepancy in acoustic neuromas(43).

In the following years, given the flurry activity in the emerging field of neurosurgery, other cases were reported, representing the first successful removal of acoustic or spinal schwannomas (44, 45); notably, it was noted that bilateral acoustic schwannomas were more frequently observed in "Central Von Recklinghausen-*disease"*, while monolateral tumours tended to occurr in (otherwise) normal patients (46).

Further knowledge on the pathology of schwannoma came from Antoni, a Sweden physician, and Pierre Masson, a Canadian pathologist. The first distinguished "Antoni A and B" fibres, which are still at the basis of the antamo-pathological classification system used in the present days: Antoni A are well organized fibers, while B fibres are more disorganized and prone to mucoid degeneration (47); Pierre Masson used for the first time the term

"Schwannomas", given the extensive evidences that these tumours originated by a degeneration of these cells.

The studies by Harvey Cushing, the famous American neurosurgeon, delayed a clear distinction between central and peripheral NF. Even if he recognized that the cellular types of Schwannomas and Neurofibromas were different, he was more prone to consider the occurrence of bilateral acoustic nerve tumours as a variant of Von Recklinghausen disease and not as the hallmark of an independent entity (48). Given Cushing's high reputation, it took almost seven decades for the two diseases to be fully separated.

One of the characteristics which continued to distinguish clearly the two forms of the disorder was the occurrence, in the central NF, of the meningiomas, which are distinct type of tumours and do not present similar histological characteristics and nerve involvement like neurofibromas or schwannomas. Meningeal tumours were present in the patient reported by Wischart, and also in the series of patients reported by the Swedish Folker Henschen (46). The same Cushing had noted a frequent association of these two "utterly different pathological character" and, in a deep revision of other reported cases (43, 45, 49), in 1938 he wrote that acoustic neuromas associated with meningiomas could represent merely gradations of the same malady and do not represent different disorders" (50).

Other groups believed that it should be considered as a distinct entity (neurofibroblastomatosis) (51), but they were isolated and cautious in their conclusions.

An important contribution in the "independence" of NF2 from NF1 came in the second half of the XX century by family studies, when some Authors noted a particular autosomal dominant inheritance, with very high penetrance (52, 53). In particular, a family, first reported in 1930, had 38 members affected over five generation, with early onset deafness, loss of equilibrium, and sometimes premature death; many of them had limited signs of the "classic NF1". The same family was at the time believed to be affected by a "central" and particular aggressive form of NF1. The "familiar" form of NF2 (i.e. spanning more than 2 generations and affecting people with milder symptoms) has been later named "Gardner subtype" after the reports of Gardner in 1933 and 1940 on families affected.

In the second part of the century, a different mutation was thought to be the probable cause of the disease: Moyes, in particular, studied 4 generation of an affected family, with few signs of the classical NF1 but a clearly distinctive onset of bilateral acoustic neuromas (54); Young et al. (55), and Kanter et al. (56) expanded the follow-up of the family reported by Gardner 40 years before (53), finding that bilateral acoustic neuromas were always present while Cafè-au-lait spot were generally small and solitary and no affected member presented

more than two spots, and other NF1 complications were notably absent, with only 2 on 97 having more than one subctutaneous nodule **[Figure 1]**.

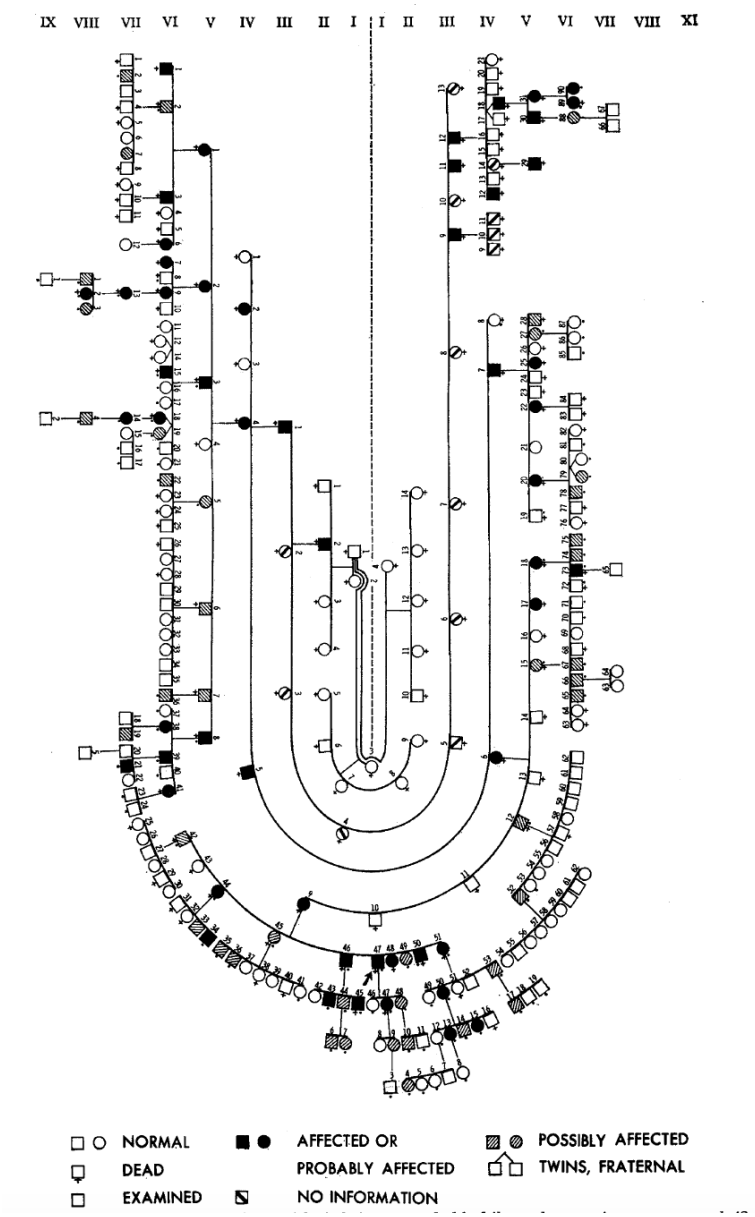

Figure 1 – The first genealogic tree of a family affected by NF2. It spans over 9 generation of people affected by the disease (18^{th}-20^{th} century)

Finally, in 1987, a consensus panel of the National Institutes of Health differentiated the clinical manifestations of the peripheral form of the disease from those of the predominantly intracranial subtype and they were subsequently named as "NF type 1" and "NF type 2", respectively. In the following years, different genetic origins of the two pathologies have been demonstrated, thus confirming the differentiation in two distinct pathologies (16, 18).

2.4 Genetic and molecular aspects of NF2

The discovery of the *NF2* gene, in 1993 (15, 57), has definitely differentiated the disease from the more common NF1, and even if the names are still similar, the conditions are totally different and, in many cases, lack common points.

NF2 gene is located at chromosome 22q12.1 (58), and encodes for a protein called *schwannomin* (or *Merlin*), which belongs to the band 4.1 family of cystoskeleton-associated proteins with several similarities with the *exrin-readixin-moesin* (**ERM**) proteins (15). These proteins are membrane-cytoskeleton scaffolding proteins, which link actin filaments to cell membrane or membrane glycoproteins. In cellular life, they have several functions: 1) to maintain normal cytoskeletal organisation, 2) to modulate cellular motility, attachment, remodelling and spreading and 3) to regulate growth (tumour suppression function) (59).

NF2 gene contains 17 exons. Northern blot analysis revealed a major 6.1-kb transcript expressed ubiquitously and a minor 2.7-kb transcript expressed in multiple tissues and several human cell lines, with some tissue also expressing a 3.9-kb transcript (60); the ratio of expression of the 6.1- and 2.7-kb transcripts are tissue specific.

NF2 transcription initiates in several possible start sites, generating at least 8 alternatively spliced isoforms. Among these, the predominant were designated as I and II (the first showing a full length, the last lacking exon 17). Other frequent isoforms carry deletion of exon 2, exon 3 or both and are expressed at low frequency (60).

The exact localization in chromosome 22q12.2 was made possible by a combined use of family linkage studies and tumor deletion mapping (61-64); the murine homologue of this gene was found later (65), located in chromosome 11, and sharing 58% similarity to the human gene (66). Curiously, in mice and men, NF2 gene is located closely to genes for leukemia inhibitory factor (*LIF*) (67) and neurofilament heavy chain polypeptide (*NFH*) (68).

2.4.1 Merlin structure and conformation

The *NF2* gene encodes two merlin *isoforms*: (**1**) the longer, dominant **isoform 1** (*merlin-1* or *merlin*), a 595-residue protein, which presents an extended carboxy-terminal tail that is encoded by exon 17; and (2) the merlin **isoform 2** (Merlin-2), which contains an alternatively spliced exon 16 which ends in a

stop codon, encoding 11 unique residues following amino acid 579 (compared to Merlin-1) (32, 69). Merlin-2 lacks the carboxy-terminal residues required for intra-molecular binding between the amino-terminal FERM domain and the carboxy-terminal hydrophilic tail, possibly leading to a constitutively *open* conformation.

The similarities of merlin to its familial group of *ERM proteins* are responsible for its cytoskeletal-binding properties: it can link the cytoskeleton to the cell membrane either directly (through integral membrane proteins) or indirectly (through membrane-associated proteins) (70).

Merlin is divided into three structurally distinct regions: (1) an amino-terminal FERM (Four-point-one, ezrin, radixin, and moesin) domain; (2) a α-helical coiled-coil domain; (3) a carboxy-terminal hydrophilic tail.

The FERM domain shares 65% sequence identity with canonical ERMs, but, differently from the other ERM proteins, merlin lacks the actin-binding site in the C-terminal domain, which is highly conserved in the other ERMs, providing these proteins with their function at the cortical cytoskeleton (71). In merlin, the binding site of the actin is located in the glutathione S-transferase N-terminal domain.

Merlin can switch from a *closed* state to an *open* state by phosphorylation at serine 518 (72). In the past, the evidence that Merlin-2 – which, as stated above, does not present the carboxy-terminal domain, thus lacking the possibility to reach a "closed" state - failed to exert tumour

suppression activity and/or contact inhibition, led researchers to state that "active" merlin functioned in a *closed* conformation caused by the dephosphorylation at Serine-518, while the *open* form [i.e., the solely form reached by merlin-2] was "inactive" and phosphorylated.

This observation has been disproved by later *in vitro* and *in vivo* studies, which demonstrated that merlin-2 inhibits cell proliferation and attenuates the downstream mitogenic signalling to the same extent of its isoform merlin-1 (thus, apparently irrespective of the open vs. closed state) (73). Several evidences now suggest that merlin exists in multiple states, which vary from "*fully open*" to "*fully closed*". The demonstration of the ability of merlin-2 to suppress growth in mammalian cell lines, has suggested that the interdomain binding is dispensable for Merlin's adhesion signalling: the phosphorylated merlin-2 displays higher interdomain binding and therefore it is able to inhibit cell growth (even) in its open state (74). Furthermore, it has been observed that a stably closed merlin mutant does not suppress cell growth, whereas merlin-2 and the S-518 phospho-deficient mutant, which are defective in interdomain binding and therefore more open, can suppress cellular growth in the same way as the wild-type merlin. It is now accepted that the "more open" forms of merlin are more active as anti-oncogenic proteins, while the "more closed" forms lack this function.

More recently, an important role has been given to the FERM domain, in particular to its "*Blue-box motif*" within the subdomain F2, correspondent to the residues 177-183 in human merlin.

By clinical observations, it is now well known that patients with truncating mutations show a more severe clinical presentation, with a higher tumour burden. However, also that missense mutations involving residues 177-183 in the F2 domain can lead to more severe forms of NF2 and it has been demonstrated that substitution of F2 domain with that of ERM protein Ezrin abolishes Merlin's anti-oncogenic activity, while substitutions of F1 or F3 domains does not affect cell proliferation (75).

In contrast, mutations involving the coiled-coil region or the carboxy-terminal tail have rarely been observed in NF2 patients, thus demonstrating the crucial role of FERM domain in the pathogenesis of the disease (76).

2.4.2 Functions of Merlin

Merlin is a powerful inhibitor of cell growth and differentiation, and can be assumed as one of the most pleiotropic anti-oncogenic proteins **[Figure 2]**. Its effects in membrane organization of proteins include cell-to-cell adhesion, cytoskeletal architecture, interaction with cytosolic proteins and regulation of different downstream pathways, including nuclear and hippo pathway regulation.

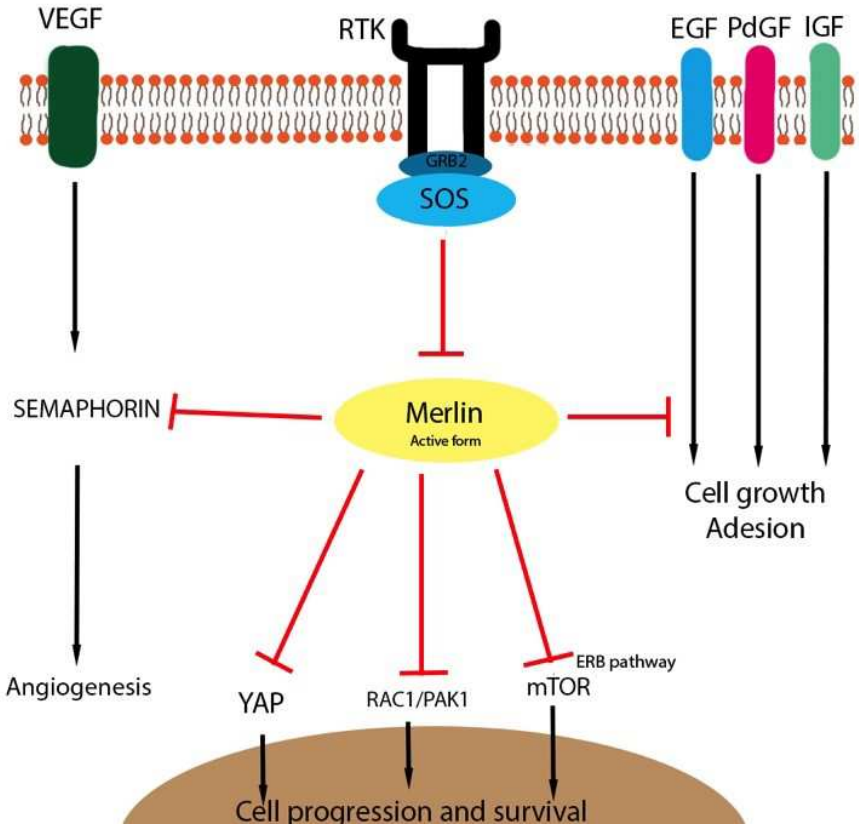

Figure 2 – Actions of Merlin as a tumour suppresor. In its active (more open) form, Merlin inhibits cell growth and cell adhesion by interfering with EGF, PdGF, IGF pathways, cell progression and survival by interfering with YAP, RAC1/PAK1 and mTOR, and, inhibiting semaphorin, reduces the VEGF-related angiogenesis.

Merlin-1 interacts with membrane associated proteins and regulates the formation of membrane domain, with a function of contact inhibition, interacting with several proteins localized in the plasma membrane, including other ERMs, the intracellular domain of CD44, α-catenin and angiomotin. Conversely, merlin-2, via the GTPase Rho/RhoKinase signalling network, promotes phosphorylation of neurofilaments that are neuron-specific intermediate filaments essential for axon structure and calibre (75).

The interactions with α-catenin have been demonstrated in keratinocites and skin epithelium: merlin promotes the binding of α-catenin and Par3, needed for the maturation of adherence junctions, and with 14-3-3 protein, which sequesters phosphorylated YAP, suppressing YAP-mediated transcription (77).

The interaction with **angiomotin** regulates contact inhibition and tumor suppression through Patj, Pals1 and Mupp1 proteins by suppressing the Rac-PAK signalling. It is important to underline that both inactive and active forms of merlin interact with angiomotin, independently from growth suppressive stimuli (78).

Other interactions of merlin occur with cholesterol-dependent membrane domains, or directly with cytoplasm proteins to control cytoskeletal dynamics, vesicular transport and microtubules stabilization, which could function in promoting the transport of anti-mitogenic biomolecules or regulating the availability of growth factors in the cytoplasm (79, 80).

In the **nucleus**, dephosphorylated merlin inhibits the pro-oncogenic $CRL4^{DCAF1}$ E3 ubiquitin ligase, even if Merlin lacks a canonical nuclear localization sequence. It is presumable that Merlin interacts with the nucleus through a motif in the c-terminus promoting nuclear export by the CRM1-exportin pathway, or by FERM domain (81).

The reduction of functions of CRL4 is mediated by interactions of merlin's FERM domain with DCAF1 (DDB1 and Cul4-associated factor 1), a substrate recognition component of the $CRL4^{DCAF1}$ complex, essential for

epigenetic modifications that regulate DNA methylation and therefore gene transcription, in particular during embryogenesis and tumorigenesis (82).

Merlin can interact also with the Hippo pathway, a potent regulator of the organs size in many species. Its disruption causes organ overgrowth and tumorigenesis, through an overexpression of YAP and TAZ transcriptional co-activator, which lead to an increased transcription of genes that promote proliferation and evasion of apoptosis (83, 84).

Despite the common action on Ras/MEK/ERK and PI3K/AKT/mTOR pathways, merlin does not interfere with learning and memory functions as it does *neurofibromin* (the affected protein in NF1) and the NF2 neurological phenotype seems, apparently, exclusively secondary to brain tumour formation and progression.

2.4.3 Regulation of Merlin

Merlin takes part to several signalling pathways, and in the recent years, all of them have been extensively characterized (85). However, its action is also regulated by several upstream pathways.

Inactivating pathways. In proliferating cells, the activation of receptor tyrosine kinases (RTKs) and Rac [Rac1/p21-kinase], stimulates PAK and leads to phosphorylation of merlin at Serine 518. This conformational change increases the interdomain binding between merlin's carboxy-terminus and FERM domain and the protein is maintained in a "more closed" (inactive) form: the

inactivation is probably due to masking of protein-interacting domains on FERM domain, which are necessary for downstream signalling or occlusion of a presumable nuclear localization signal (86). Apart from Rac and PAK, also Protein Kinase A enzymes can phosphorylate merlin at Serine 518 and 10 (87) and this is relevant in Schwann cells, which are sensitive to the cyclic AMP-PKA signalling axis (88). Protein kinase B, also named AKT, can induces phosphorylation at threonine 230 and serine 315, causing decreased interdomain binding, and the interaction with Phosphoinositide and PIKE-L (89).

Activating pathways: In contact-inhibited cells, dephosphorylated (active) merlin accumulates as a result of intercellular adhesions, which lead to PAK inhibition (75, 90). Different phosphatases may contribute in dephosphorylating merlin, but the key role is played by cadherins and CD44 receptors, which activate merlin through its dephosphorylation by myosin phosphatase targeting subunit 1 (MYPT1) (89). This last enzyme is inactivated by protein kinase CPI-17 (c-potentiated phosphatase inhibitor 17 kDa weighed) (91). By contrast, cadherins cause loss of function of PAK protein, thus reactivating Merlin.

2.4.4 Genotype-phenotype correlations

NF2 is inherited as an autosomal dominant trait with nearly 100% penetrance by 60 years of age (92). There is a relatively strong genotype/phenotype correlation both with type and position of *NF2* germline mutation (93, 94).

Most of the mutations occur de novo, as germline mutations. Is it not known the prevalence of mutations inherited by parents with somatic mosaicism, but the figures of patients diagnosed with NF2 harbouring a mosaic mutation span from 30% of those with bilateral acoustic schwannomas, to 60% of patients with a monolateral schwannoma (95): this is not surprising, as many neurocutaneous disorders are nowadays thought to be the result of a somatic mosaicism (1).

Mutations in *NF2* gene can be missense (alteration of a single base with substitution of a single aminoacid of the protein), nonsense (with a premature truncation of the protein in any codon of the gene), frameshift (involving the deletion of a number of bases different than 3), in-frameshift (deletion of three bases and skipping of one aminoacid). In many patients, large deletions involving the whole gene or entire exons have been reported.

Missense mutations are associated with a considerable lower risk of mortality and a milder NF2 phenotype (96, 97), especially if they are present in less than 100% of cells (mosaicism). Nonsense mutations and frameshift mutations, generating a premature truncation of the protein, are associated to worse prognosis (98): individuals presenting such mutations have earlier onset of symptoms, more meningiomas and spinal tumours and die at younger ages. Truncating mutations involving exons 14-15 and, surprisingly, exon 1, are associated with a better prognosis (99).

The effects of deletions are still matter of study: one can think that larger deletion would be associated with a more severe phenotype, but this is not universally true, with some of them producing an unexpectedly mild phenotype (99). Splice-site mutations can be associated with several phenotypes, with higher mortality for mutations occurring in exons 1-5 and in exons 11-15.

Germline mutations occur throughout the first 15 exons but not in exons 16 and 17 of *NF2,* which encode for the last parts of the α -helical domain (100) ,and the predominant mutations are nonsense at CpG sequences.

2.5 Clinical Features

NF2 is a chronic condition associated with considerable morbidity, reduced quality of life and life expectancy (101): before the use of selective anti-neoplastic drugs and improved surgical techniques life expectancy was estimated in 36.25 years (102). The main symptoms of NF2 are related to the uncontrolled growth of benign tumours in central and peripheral nervous system, which are also the cause of the high morbidity and mortality presented by individuals affected by the syndrome.

Symptoms onset is usually comprised within 2 and 20 years of age, and diagnosis is usually delayed (5 to 10 years). Most patients present hypoacusia or deafness, loss of equilibrium, uncertain gait as their first symptoms.

Bilateral vestibular schwannomas (VSs) are the distinctive feature of neurofibromatosis type 2 and are present in 90-95% of cases, even if they may be not the first tumour to appear, especially in pre-pubertal patients. These tumours are not malignant (i.e. they do not metastasize, do not present a rapid and uncontrolled growth, are well limited to the VIII cranial nerve and do not infiltrate the surrounding tissues), tend to have a multifocal origin within the nerve (103), but the location near the brainstem (pons) and in the temporal lobe of the brain are related with high morbidity and mortality (104) [**Figure 3**].

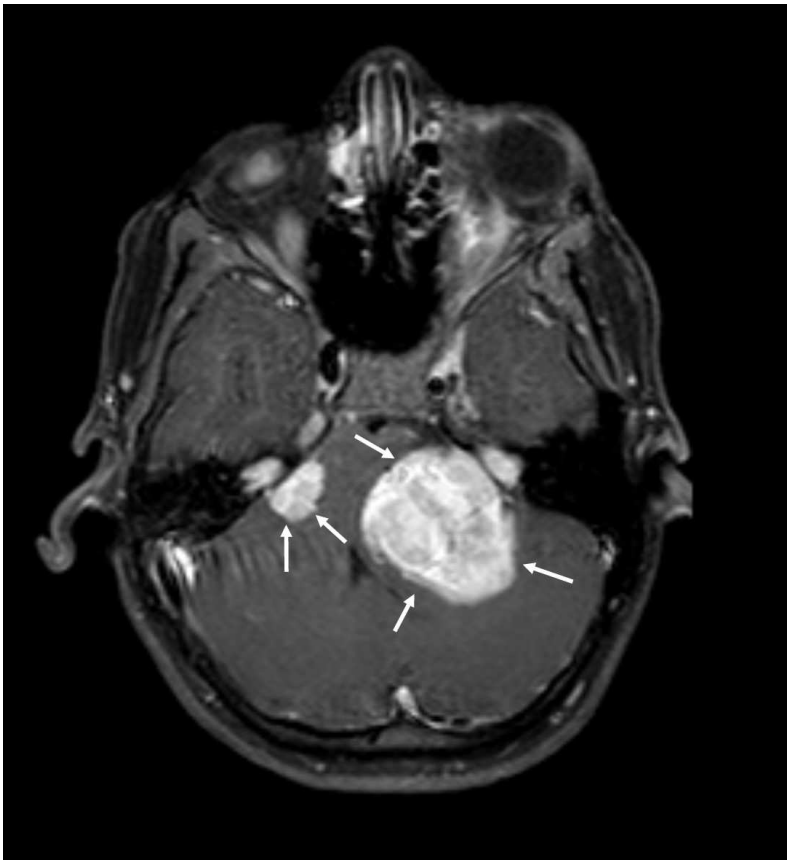

Figure 3 – Axial, contrast-enhanced T1 MRI of a patient affected by congenital NF2 showing bilateral VSs (white arrows)

The earliest consequence of such tumours growth is represented by hearing loss and tinnitus, which in many cases are unilateral at onset (depending on which of the two nerves is involved earlier). In paediatric patients, hearing loss is the presenting symptom only in 3% of cases, mainly because the children may not be able to refer this symptom. Children show a higher growth-rate of these tumours, and congenital forms are usually the most severe (32, 105, 106); tumour growth varies among patients and even within the same families (107, 108).

Aside the VIII, schwannomas can be present in all the other cranial nerves, with the exception of olfactory and optic nerves (I and II) that lack of Schwan cells, as well as in spinal and peripheral nerves. Up to 50% of patients presents a schwannoma in cranial nerves III, V and VII, usually asymptomatic. The fewer patients with lesion involving the lower cranial nerves present a more pronounced symptomatology.

Another typical hallmark of the disease are the **meningiomas**, the second most common tumour in NF2. About half of the patients (45-58%) present an intracranial meningioma [**Figure 4**] and about 20% intradural extramedullary spinal meningiomas: both the tumours originate from the same meningeal tissue and may be considered as a unique entity (109, 110). In the general population, it has been estimated that up to 20% of children presenting with a meningioma is affected by NF2 (104, 111).

Meningiomas tend to occur as multiple lesions, most of the time without symptoms before reaching large dimensions; when they are located in optic nerve sheath, skull base, spinal canal the onset of symptomatology may otherwise be precocious, with reduced vision or blindness, headache, autonomic symptoms. The occurrence of meningiomas in NF2 patients is related to a worse prognosis, with a 2 to 5-fold increased mortality risk (112), probably because, differently to what observed in the general population, NF2-related meningiomas have a higher proliferative activity and a greater amount of anaplastic or atypical cells (113).

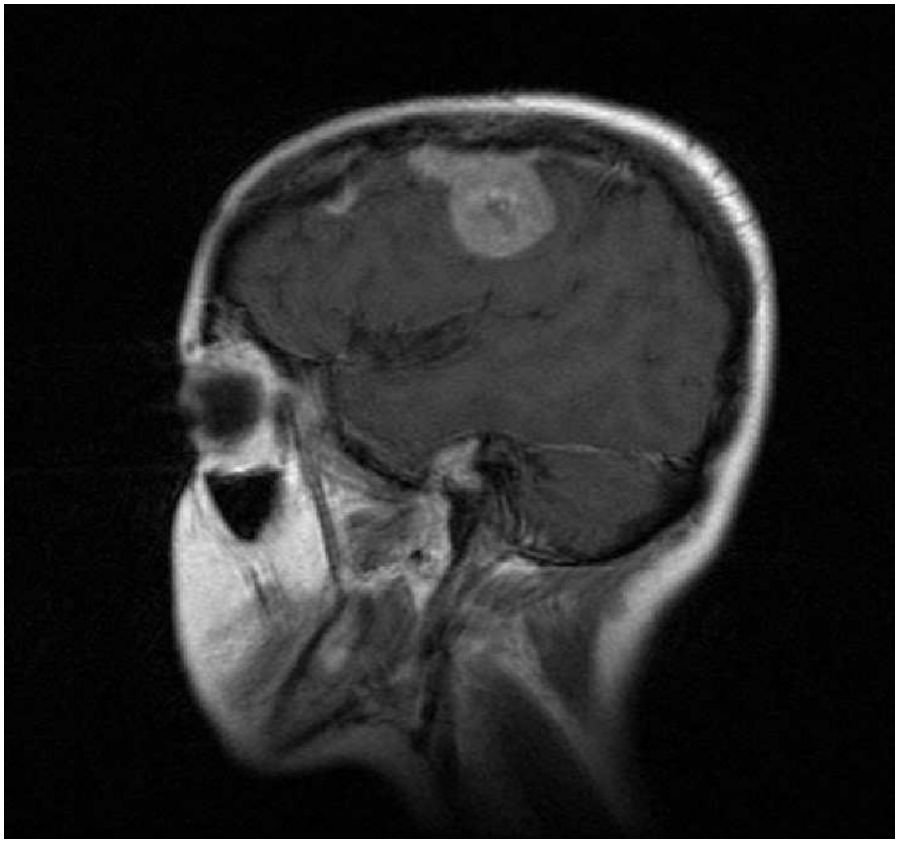

Figure 4. Sagittal, contrast-enhanced T1-weighted Magnetic Resonance Image of a NF2 individual presenting a meningioma. In the initial phases, symptoms may be absent

Moreover, in most of the cases, they cannot be fully eradicated and high-dose radiation therapy is associated with several effects, such as neoplastic degeneration or formation of adjacent tumours (114).

Intramedullary spinal cord tumours are in 3 cases out of 4 **spinal cord ependymomas**, which are present in up to 50% of patients, and cause symptoms in less than 25% of cases, mostly back pain, weakness or sensory disturbances (115). Surgery is performed only in 20% of patients, as the most part of these tumours tend to remain quiescent (116). The most common site of involvement is the cervical cord or cervicomedullary junction **[Figure 5]**, followed by the thoracic and lumbar cords.

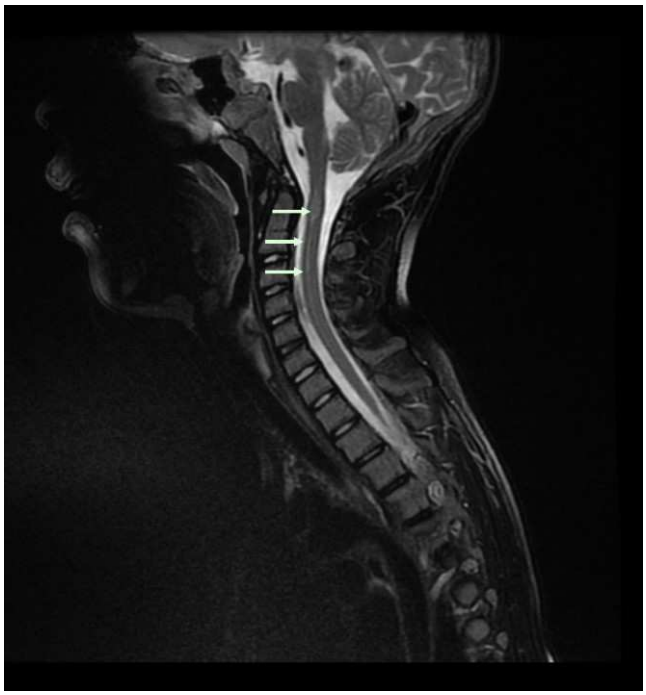

Figure 5. Sagittal T2-weighted [**A**] magnetic resonance images of the spinal cord, showing a long rounded lesion (green arrows) within the cervical cord: this intraspinal ependymoma occurred in an NF2 child who had upper limb paraesthesia and motor deficits.

Nonsense (truncating) and frameshift mutations (those related with a worse prognosis) are present in 60-70% of patients affected by spinal cord ependymomas (115). More rarely, intramedullary tumours can be astrocytomas of the spinal cord (diffuse or pilocytic) or schwannomas (117).

Extramedullary tumours are represented in almost all the cases by schwannomas of the spinal nerve root, which have to be eradicated in up of 30% of patients. Schwannomas can involve all the peripheral nerves, as well as the subcutaneous tissues (these last lesions are very sensitive to pressure and related to severe pain) (118).

A typical finding of the syndrome is represented by **chronic peripheral neuropathy**, which can be related or not to the growth of tumours in surrounding nerves: up to two third of the patients have a peripheral neuropathy in absence of tumours, and in many cases a focal neuropathy can precede the onset of the tumour (119). An intra-nervous accumulation of non-compressive fascicular lesions is thought to be the cause of the development of this manifestation, as well as cumulative compression by multiple, small, undetectable tumours along the peripheral nerves or local toxic or metabolic determinants or loss or dysfunction of Schwan cells (104, 120, 121). Peripheral neuropathy manifests with focal amyotrophy, distal symmetric sensorimotor neuropathy, or mononeuropathy multiplex, with age of onset ranging from 5 to 40 years, and duration ranging from 3 months to 50 years (122-125).

More rarely, patients may present intracranial calcifications, dysplastic foci in the cerebral cortex and basal ganglia and meningoangiomatosis (126-128)

Ocular involvement is particularly frequent in NF2 patients, represented, in particular, by lens opacities **[Figure 6]**: a cataract manifesting before 50 years of age is typically due to NF2, and is usually located in the posterior subcapsular, capsular or peripheral cortical regions (26, 129).

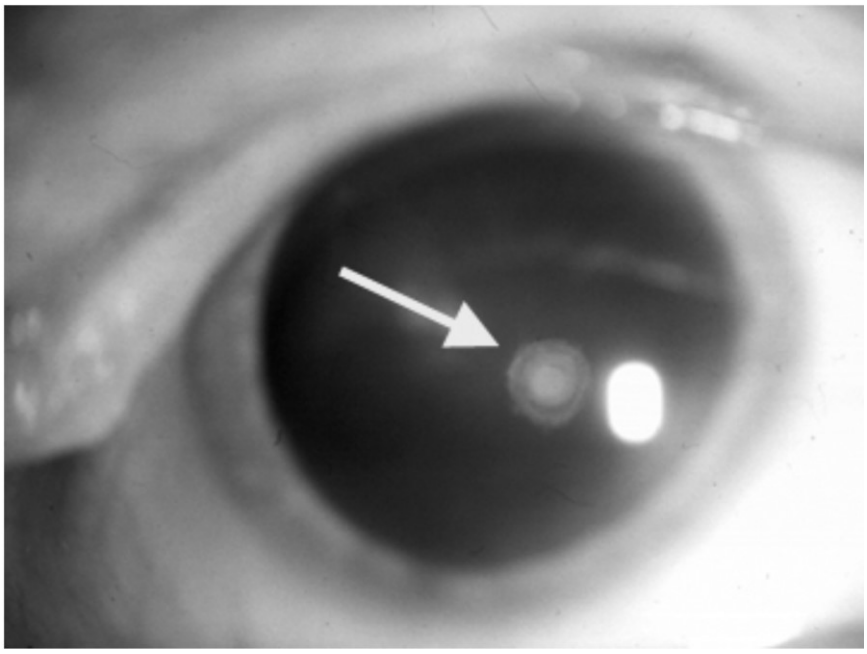

Figure 6. Magnified view of the eye in an NF2 child showing subcapsular lens opacities (white arrow)

The impairment of vision in patients affected by NF2 can be equally related both to cataract and to meningiomas affecting the optic nerve sheath. Other ocular manifestations are epiretinal membranes and retinal hamartomas, which rarely cause loss of visual acuity (130, 131).

Differently from NF1, **cutaneous manifestations** of café-au-lait type are rarely observed and in a minor number (up to one-two spots per patient). Only 1% of patients presents more than six cafè-au-lait spots (102). More commonly, at least in 50% of individuals, skin tumours may be present, and these include skin plaques **[Figure 7]**, subcutaneous and intradermal tumours, most of which are schwannomas (132).

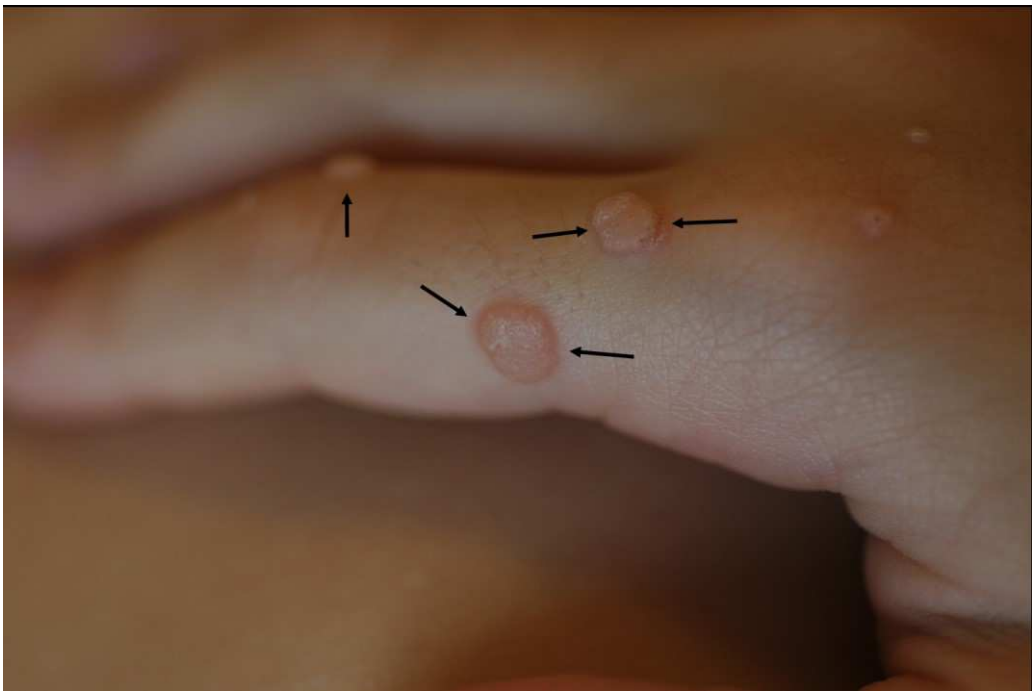

Figure 7. Close view up of the skin showing classical NF2 plaques (in atypical locations) over the fingers (black arrows) of a child with "congenital" onset NF2.

The plaques are well circumscribed, only slightly raised, typically smaller than 2 cm and with a minor hyperpigmentation or hypertrichosis (104), while subcutaneous tumours develop along the peripheral nerves, can be appreciated by palpation or observed as fusiform or nodular swelling, and are

painful and sensitive to pressure; intradermal tumours are rare and appear as epicutaneous, well demarcated soft lesions (133, 134).

2.5.1 NF2 in childhood: Congenital and *Wishart* subtype

NF2 with onset in childhood has been identified for centuries as "Wishart subtype", after the first physician who described such a (severe) phenotype in the early XIX century (30), while the milder phenotype, with a typical onset in adulthood, has been termed "Gardner subtype" (45), after the first report of (large) familial cases with autosomal dominant inheritance but still preserved ability to reproduce (late-onset subtype).

In childhood, NF2 has a particular course, usually severe and complicated by tumours other than the bilateral acoustic schwannomas (i.e. meningiomas, spinal cord tumours) that may present a rapid course. At least 18% of NF2 individuals have a disease onset before the age of 10 years (135, 136).

Congenital NF2 presents within the first year of life (106). It is characterized by the occurrence of small (< 1 cm) bilateral VSs that are usually detected incidentally by MRI, as they tend to remain asymptomatic till the second decade of life, when they show a rapid progression. The presence of such tumours in very early life allows to consider those as "congenital", similarly to what happens for optic pathways gliomas in (some) NF1 patients or giant cell astrocytomas in some patients with tuberous sclerosis complex (2).

Skin abnormalities are very common, and in particular NF2 infants may present a large number of NF2 plaques in atypical locations such as face, hands, knees; concurrently to the progression of VSs, NF2-plaques tend to reduce in size and number during the teen-age. Cafè-au-lait spots may present with irregular margins and with large dimensions. Lens anomalies appear in the first months of life, as well as asymptomatic cortical malformation and/or diffuse high signal lesions at brain MRI in the periventricular regions, thought to be meningioangiomatosis. Congenital NF2 arises by de novo mutations and the disease usually does not affect the parents (106).

Childhood-onset (Wishart subtype) NF2 is usually severe. In the pre-pubertal NF2 age group, subtle skin tumours, small posterior capsular or cortical edge cataracts or neurological signs secondary to other-than-VSs cranial nerve(s) involvement and/or brainstem or spinal cord compression are more common and manifest years before dysfunction of the eight-cranial nerve (26).

Skin manifestations encompass few café-au-lait spots and/or peripheral nerve tumours, much similar to the cutaneous neurofibromas observed in NF1 patients (for this reason they are often diagnosed as having NF1, in a first phase). The limited number of cafè-au-lait spots, their irregular margins and the lighter colour, are usually less distinctive, and for this reason (children) patients are usually discharged as non-having NF1, with a diagnosis of NF2 being suspected later, only after other signs begin to appear. NF2-plaques (histologically schwannomas) are characteristic, being slightly raised and

pigmented in colour, usually <2 cm in diameter. Together with the spherical subcutaneous nodular tumours they can be present in up to 90% of NF2-children and they are located mainly in upper and lower limbs (26, 137, 138). Ophthalmologic manifestations tend to be quite common (35-70%) and are represented mostly by juvenile posterior subcapsular or cortical cataract and retinal hamartomas. They are usually asymptomatic, manifesting only when their dimensions are large (approximately 20% of NF2 patients) (138). Amblyopia and strabismus may be present and in most of the times related to a further development of tumours in third, fourth, sixth cranial nerves (all controlling the movements of the eye), as well as skull base, brainstem and facial nerve (26).

Other neurological manifestations are strictly related to the CNS tumours, but usually spare (in the first phases) the auditory and vestibular functions (136), but not the other cranial nerves (with deficits of their function, including autonomic dysfunction, strabismus and amblyopia, control of facial muscles), the brainstem or the spinal cord (focal muscle wasting).

The presence of meningiomas is usually more common than in adult population, but, as in adulthood, rarely related to specific symptoms: epilepsy and/or headache are rare in childhood, as well as meningioma-related deafness (139).

2.5.2 Mortality in NF2

Patients affected by NF2 have diminished lifespan compared to non-affected family members (99, 112). The overall survival rates at 5, 10, and 20 years after diagnosis are 85%, 67%, and 38%, respectively (140). As above specified, the worse prognostic factors are an early age at diagnosis, the presence of intracranial meningiomas and truncating or splice-site [exon 1-5] *NF2* mutations. Patients affected by mosaicism have a better prognosis, as well as patients treated in tertiary care centres or harbouring a splice-site [exon 6-15] or missense mutation. The most frequent causes of death include tumour burden, peri-operative complications, and malignancy from NF2-related tumour (141). Notably, the overall mortality of children and adults with NF2 diagnosed in more recent decades has been dramatically reduced, given the improvement in surgery, radiation and the introduction of new pharmacological agents (99).

2.6 Diagnosis

The first Clinical criteria for the diagnosis of NF2 were established at the National Institutes of Health (**NIH**) Consensus Conference on NF1 and NF2 in 1987 (142) and revised in 1990 (143). The presence of bilateral VSs was almost mandatory for the diagnosis of NF2, with the exception of those with unilateral VS born by a parent with a diagnosis of NF2 and presenting other tumours typically occurring in NF2. For these diagnostic criteria, however, patients without bilateral VSs or without a family history of NF2 cannot qualify for a

diagnosis of NF2. This was (and still is) particularly relevant for childhood NF2 whose, initially and during part of their natural, progress without eight-nerve dysfunction and often lack affected members in the family because of the high occurrence of sporadic mutations.

Revised criteria were proposed by the *Manchester group* in 1992 (102) and by the National Neurofibromatosis Foundation (**NNFF**) in 1997 (144), with the aim to include in the diagnosis all the patients with features associated with NF2 and who did not reach the strict NIH criteria.

After the discovery of the causative gene, many doubts on the diagnosis were cleared, even if in many cases a mutation cannot be exactly identified or its pathogenicity clarified. Moreover, NF2 can occur in alternate forms, even if rarely in childhood, such as mosaic/segmental NF2, schwannomatosis, multiple meningiomas (1)

The most recent diagnostic criteria have been developed by Baser et al, who attempted to encompass all the above problems by empirically developing and testing a scored set of diagnostic criteria (145, 146) that uses current understanding of the natural history and genetic characteristics of NF2 to increase sensitivity, maintaining at the same time very high specificity: these criteria currently permit early diagnosis in a greater proportion of patients with NF2 than previous sets of diagnostic criteria **[Table 1]**. The main clinical characteristics are a first-degree relative with NF2 (family history), unilateral (low score) or bilateral (high score) VSs, a meningioma (with a higher score if a

second meningioma is present), cutaneous schwannomas or other cranial nerve tumours, and mononeuropathy and cataract as other symptoms. If these symptoms are present before the age of 30, the score is higher: the peak of incidence of NF2 is in the first three decades of life, and after this age tumours tend to present as sporadic or secondary to other causes (environment, other genetic conditions).

2.7 Management of NF2 patients

Initial evaluation of children who have or are at risk for NF2 should include testing to confirm a diagnosis and to identify potential problems, including in particular tumours surveillance (147)

When asking for the medical history, physicians should focus their attention on focal neurologic symptoms, skin tumours and/or cutaneous spots, headache, and visual symptoms as well as auditory and vestibular function. Seizures are rarely observed in patients with NF2, especially in the first years, but can occur as a presenting sign of increased intracranial pressure or brain tumours. It is strictly recommended to focus the attention on hearing problems in the first-degree relatives as well as neurological problems presented by the members of the family (133).

As soon as possible, an MRI scan of the brain must be performed, and include gadolinium-enhanced axial and coronal thin cuts (1-3mm) through the brainstem to identify VSs (26). Cervical spine should be investigated for the

occurrence of ependymomas or meningiomas/schwannomas in the paravertebral regions. It is still debated whereas MRI should be extended also to thoracic or lumbar spine: some Authors prefer to investigate these regions routinely, while others only if neurological symptoms referable to these locations are present (104). In any cases, a complete neurological examination serves as a baseline for evaluating the evolution of the diseases and may assist in the selection of sites within the nervous system that require further imaging studies.

Ophthalmologic examination permits to identify characteristic lesions of the eye, including lens opacities, retinal hamartomas, or epiretinal membranes (132).

Audiology (including pure tone threshold and word recognition) and brainstem auditory evoked responses (BAER) document dysfunctions in the eighth cranial nerve related to VSs and are mandatory to characterize the course of the disease. Abnormalities of pure tone thresholds may be present in up to 90% of subjects between 10 and 72 years. Word recognition measures the functional hearing, while BAERs are a more sensitive measure of auditory function, being abnormal in 100% of patients with symptomatic VSs.

Biopsy of cutaneous anomalies is reserved only to uncertain cases, as their discovery in a patient with a history of bilateral VSs is sufficient enough to clarify their nature. However, in most of the paediatric cases, the occurrence of solitary cafè-au-lait spot usually precedes the brain tumours and for this

reason patients, in the very first stages, can be misdiagnosed as affected by other neurocutaneous disorders.

Follow-up is extended to all the patients' life, as there is no definitive cure for the disease. After initial diagnosis, children should be visited very frequently (every 3-6 months) until the growth rate and biologic behaviour of tumours is determined (this is particularly important in congenital NF2). Consultation with an experienced surgeon after initial diagnosis is often helpful for patients with adequate hearing to discuss the feasibility of hearing-sparing surgery, which can be possible especially when tumour masses are not large.

Most paediatric patients without acute problems can be visited every 6 months or with an annual basis. Evaluation should include complete neurological examination, MRI scans of the brain and spinal cord with thin cuts through the brainstem, MRI scans of symptomatic lesions outside the brain (if present), audiology, and BAER. Yearly audiology documents changes in pure tone threshold and word recognition, a very helpful information in planning early surgical intervention for VSs and in counselling patients about possible deafness. Changes in BAERs usually precede hearing loss. Ophthalmologic evaluation should be performed in selected patients who refer visual impairment, or present involvement of ocular cranial nerves (strabismus, facial weakness).

2.8 Genetic counselling

Individuals at risk for NF2 (especially those with one parent affected by the disease) need genetic counselling and pre-symptomatic testing. Clinically, a normal MRI at 16 or 18 years reduces the chances of having inherited NF2. A normal MRI at 30 years makes inheritance very unlikely, except in late-onset families, and may justify cessation of formal screening (148).

As clinical and radiological screening cannot reassure at-risk individuals that they have not inherited the condition until 30 years of age, molecular genetic testing for NF2 has had a great impact on clinical practice (100): constitutional mutation testing for NF2 by Sanger sequencing or WES-based panels are now available worldwide and mutations found in about half the sporadic individuals tested and in over 90% of families with vertical transmission (95).

2.9 The "classical" treatment of NF2

In disorders like NF2, characterized by the occurrence of multiple tumours for all the life, the approach cannot be characterized by surgical removal of all the lesions, as it would not be possible or advisable. The primary goal has always been to preserve the functionality, to minimize the symptoms or the deficit and to maximize the quality of life (149, 150).

Currently, surgical removal remains the standard therapy for VSs and other brain or spinal tumours: it has to be noted that patients undergoing

surgery for VSs often experience iatrogenic hearing loss in the treated ear requiring rehabilitation through the use of a cochlear implant or an auditory brainstem implant (**ABI**) (151-153). As an alternative or in addition to tumour removal, stereotactic irradiation and/or chemotherapy can be used to delay tumour progression, but they have been associated to the development of secondary malignancies(154), and, in particular radiation therapy, can accelerate loss of hearing (155, 156). Complete surgical resection is curative, but the timing for tumour removal is controversial: risk-benefit ratio must evaluate accurately the side effects related to surgery and tumour's natural history (108).

2.9.1 Surgical strategies

Especially in childhood, tumours have a very high growth rate and can be present in any body district, in particular skin, brain and spine, as well as in peripheral nervous system (32): when the management of these patients is performed in multidisciplinary specialty treatment centres there is a significantly lower risk of mortality than in those patients treated in non-specialty centres, with higher rates of favourable outcome in VSs surgery and lower rates of serious complications with increasing surgical experience (134).

Dermal tumours are rarely treated in childhood, mostly because of cosmetic impact (135); their growth, in fact, tends to be slow, in line with the growth patterns of peripheral schwannomas, which rarely are disfiguring in

younger ages. (136) Interestingly, resection of typical NF2-plaques is being asked for diagnostic clarification or biopsy, as often, in the paediatric population VSs are not present and diagnosis is doubt (26).

When brainstem or spinal cord compression occurs (usually as a consequence of large VSs or other intracranial tumours), surgery is clearly indicated, as well as in case of obstructive hydrocephalus. If little or no neurological dysfunction is related to the tumours, a watchful waiting may allow children to retain neurological and auditory functions for several years (157).

Paediatric NF2 patients have often, in the first phases of their disease, one or more extradural spinal masses (schwannomas or meningiomas), which rarely cause symptomatology or impairment of spinal functions (158). In the case of spinal ependymomas (intradural tumours), surgery is usually performed with success and no needing of repeated intervention and no risk of regrowth (115).

Given their threatening localization, brainstem tumours of the pons or the bulb (ependymomas and astrocytomas) most often need repeated partial resection with severe sequelae. Indications for resection include rapid tumour growth and worsening neurologic symptoms, as these tumours' location in NF2 is associated with higher-grade histology, relentlessly progressive course and often a fatal outcome bench (32).

The typical findings in NF2 patients are bilateral VSs and surgical

strategies on their management have been widely debated: till today, the decision to operate early or wait until the first symptoms (hypoacusia or BAER anomalies) is difficult. Pre-operative tumour size as an indicator of outcome in VSs surgery is still debated. In a consensus paper, surgical resection was proposed only for tumours larger than 3 cm in diameter (159), thus permitting facial nerve preservation and brainstem protection, while there is no standardized surgery for VS less than 3 cm in diameter, which often are not associated with symptoms. In these cases, pre-symptomatic surgery aims to retain hearing with a minimum of post-operative complications such as facial weakness or dysphagia.

In patients with a documented change in hearing, bony decompression of the internal auditory canal through a middle cranial fossa can stabilize hearing for a period of time (160-162), but are not definitive. A sub-occipital approach for small tumours can result in hearing preservation although only few reports using this technique have been published (163). The trans-labyrinthine approach with placement of an auditory brainstem implant can provide auditory sensations in some patients, even if 10% of patients do not receive auditory sensations, and, when present, the quality of sound is often low and the processor optimization requires regular follow-up (152, 153, 164, 165). A further option is cochlear nerve-preserving surgery when the tumour is small via the translabarynthine approach and insertion of a cochlear implant that produces far superior results to ABI (166). At the present time, little

information is available about the efficacy of hearing sparing surgery in paediatric NF2 patients, but outcomes appear to be inferior to that for adults (138).

Indications for surgical resection of other cranial nerve schwannomas are less well defined. In general, schwannomas of other cranial nerves present slow growth and produce few symptoms: surgery is reserved only for those with unacceptable neurologic symptoms or rapid tumour growth (32).

2.9.2 Radiation therapy

Radiation has been often used as adjuvant therapy for treatment of sporadic brain tumours, and in NF2 outcomes have been showed to be worse than in general population. Usually, surgery is preferred in a first stage and radiation treatment reserved for tumours that are not surgically accessible, also because radiation therapy makes subsequent resection of VSs and ABI's implant more difficult.

In early studies of stereotactic radiosurgery, 18-20 Gy were delivered to tumours, with optimum results in terms of tumour control (more than 90% of patients), but loss of auditory functions in all the patients. In the following years, the dose of radiation was reduced to 12-16 Gy and with the improvement of stereotactictic radiosurgery, tumour control can be achieved in more than 95% of patients with preservation of hearing in 40-67% (167). The risk of deafness in patients with pre-operatively serviceable hearing is about 20%, and

among the other side effects, a decreased facial and trigeminal function occurs in 5-16% and 10% of patients, respectively(168). More recently, fractionated stereotactic radiotherapy has been advocated to minimize the risk of hearing loss, with efficacy in tumour control in 93% and a hearing-preservation rate of 64% (169). Radiation therapy, especially in childhood, is conversely associated with a greater risk of induction or malignant transformation (170).

The role of adjuvant radiation in other tumours such as meningiomas and ependymomas is not established, as the majority of these tumours demonstrate benign histology and can be controlled surgically. Only sporadic cases have been published on treatment of NF2-related meningiomas (171). Control has been achieved in almost all the cases, with peritumoral oedema occurring in 25% of patients and cerebral oedema in less than 10% (156).

2.9.3 First chemotherapy attempts

Before the introduction of monoclonal antibodies and biologically targeted drugs, there was no effective chemotherapy for treatment of NF2-related tumours. Given the complexity of the disorder, and in particular the severity of VSs, chemotherapy was reserved only for refractory meningiomas or non-surgical tumours.

Hydroxiurea was initially used in the treatment of meningiomas, but without confirmation of its efficacy in the following years in clinical practice, despite a cytostatic effect in vitro (172). In the same study, no major responses

were detected to other alkylating compound such as erlotinib, mifepristone, losartan, metformin and verapamil (172).

No trials of chemotherapy for treatment of vestibular schwannomas or ependymomas have been reported. Gene therapy remains a potential option for the future as injection of oncolytic recombinant herpes virus into schwannomas in mice resulted in significant tumour shrinkage (173).

2.10 Biologically targeted anti-neoplastic drugs

Until the last decade, surgery, radiation therapy and the (few) antineoplastic drugs were the only therapeutic options for NF2-related tumours. More recently, biologically targeted therapies using molecules driven from the increased knowledge of the molecular pathways involved in the pathogenesis of NF2 have been successfully employed in adult and paediatric patients (133). These drugs have been tested only in a few patients, given the rarity of the disorder and the lack of data on their tolerability and efficacy. Such protocols, in many cases, have obtained a tumour volumetric shrinkage, an arrest of progression of tumours or a reduction of time to progression, as well as results in hearing function. Given the complexity of the Merlin's pathway(s), different targets have been proposed.

Antagonists of the ErbB family. **Lapatinib**, an antagonist of the Her1-2 members of the ErbB family of tyrosine kinase receptors, was clinically tested in 17 NF2-patients, with volumetric regression of VSs, in 4 out of 17 and an

appreciable hearing response in 4 out of 13 evaluable patients. Toxicity was minor with no dose adjustment required (174). More recently, a combination protocol of lapatinib or nilotinib with radiation therapy has achieved appreciable results in an *in vitro* NF2 model (175). On the other hand, among eleven patients treated with another ErbB tyrosine kinase receptor inhibitor, **Erlotinib**, only three showed a mild tumour shrinkage (4 to 14%); hearing evaluation was performed in 9 patients with one showing a transient response, 2 a minor response, 3 remaining stable, and 2 developing progressive hearing loss (176).

Inhibitors of the mTOR pathway. The attempt to use mTOR pathway inhibition (i.e., **Everolimus)** did not yield appreciable results, with no patients (out of 9 enrolled) showing a volumetric response of the schwannomas, nor clear evidence of a stabilization of the disease (177). In another study, five out of 9 patients showed a stable disease (tumour progression decreased form 67%/year to 0.5%/year) and a rapid re-growth after treatment discontinuation (178). In none of the study severe side effects occurred. Recently, in a 60-year old woman affected by urethelial cancer showing a NF2-causing mutation, the use of a combination of everolimus and paclitaxel was associated to an appreciable volumetric regression (179).

Inhibitors of IGF1 receptor/PDGF/Akt/MEK. In vitro studies on different potential drugs have shown results in terms of tumour shrinkage, in particular with **picropodophyllin**, an anti-IGF1 receptor, (180), **OSU-03012**, an Akt

inhibitor (181), and **nilotinib**, a PDGF and C-kit cascade inhibitor (182, 183). This last drug has been also tested in combination with radiation therapy. Another PDGF inhibitor, **imatinib**, has also been employed in a 30-year old NF2 patient: the preliminary results were appreciable (he showed an arrest of growth for 4 months), but the treatment was suspended due to the occurrence of severe adverse reactions, including headache, vomiting, abdominal pain and increased unsteadiness. The same patient was then treated with bevacizumab, showing appreciable results (184). Also **Sorafenib**, which acts by inhibiting C-kit, PDGF and MEK1-2 system, was employed in a single patient and afterward suspended because of the appearance of a severe adverse reaction (diffuse rash) (185).

2.10.1 Anti-VEGF therapy: Bevacizumab era

Bevacizumab is a monoclonal antibody directed against the anti-vascular endothelial growth factor (**VEGF**). Since its first employment by Plotkin et al. in 2009, it has shown the most promising results in terms of tumour volume shrinkage, stabilization of the disease and hearing improvement, at a dose, initially, of 5 mg/Kg/biweekly (186) **[Table 2]**. It has been showed to be well tolerated in NF2 patients and the side effects occurred, which include haemorrhage, delayed wound healing, proteinuria and hypertension, have rarely caused a cessation in the treatment (187, 188).

Vestibular Schwannomas: Regarding the VSs, after the first experience in 2009, patients were evaluated again after 3 years: a stable or improved hearing was observed in 90% of patients after 1 year and 61% after 3 years; a stable or decreased tumour volume was observed in 88% of patients after 1 year of treatment and in 54% at 3 years (189). In the same years, in a small study two patients showed a reduction of more than 40% of the volume of the tumours and a substantial hearing response after only 6 months of treatment (190). Higher dosages (10 mg/kg/biweekly, than 15 mg/kg every third week) were related with appreciable results in the experience of Alanin et al in 12 NF2 patients (191): a radiological response (> 20% tumour shrinkage) was observed in seven out of 18 tumours (39%) in six out of 12 patients (50%) and sustained radiological responses were maintained in six tumours (33%) for more than 2 months. Three patients (25%) had an improvement of hearing and five patients (41.7%) reported subjective benefit in neurological symptoms, including improved hearing. Toxicity was in general manageable, but one patient died from cerebral haemorrhage (possibly related to therapy). A reduced dosage of 2.5 mg/kg/biweekly was however reported as useful by Farschtschi et al. in 3 patients, with a lower occurrence of side effects (192), and in a 55-year old patient who showed a marked tumour reduction (193); moreover, in order to further reduce the side effects and to optimize the treatment efficacy, a super-selective infusion of bevacizumab in the tumour has been also proposed (194).

More recently, a protocol with a "standard" dosage of 7.5 mg/kg (every three weeks) has showed that a hearing response improvement was maintained for more than 1 year in five (36%) of 14 patients, while eight (57%) had transient hearing improvement; notably, no patients experienced hearing decline. Radiographic response was seen in six (43%) of 14 target VSs. Among the side effects, three grade 3 reactions occurred (two cases of severe hypertension and one immune-mediated thrombocytopenic purpura) (195).

Meningiomas: After a promising anecdotal report showing a marked decrease of a intracranial meningioma in a 55-year old man (196), a minor efficacy was demonstrated in the study by Nunes et al., with a volumetric response observed in 29%, a median duration of the response of 3.7 months and a median time to progression of 15 months (197). However, in a further study by Alanin et al. (198) on 14 intracranial meningiomas in 7 NF2 patients, treated with 10 mg/kg every two weeks for six months and 15 mg/kg every three weeks thereafter), a decrease in volume was observed in 5 of 14 meningiomas (36%) in 5 of 7 patients (71%), with a median decrease of 10% (range 3%–25%); only one meningioma (7%) progressed and the remaining (93%) were stable.

Spinal cord ependymomas: More recently, bevacizumab efficacy has been evaluated also in these tumours, after the case of a 28-year old woman showing benefits from the treatment (199). In the study by Faratschi et al. in eight

patients, a clinical response (pain relief, improvement of neuropathy) was observed also in patients who did not show any radiologic response (200); this results were confirmed by Morris et al. (201), who observed a clinical improvement only in patients affected by cystic ependymomas: bevacizumab treatment resulted in a decrease in the size of intratumoral and juxtatumoral cysts as well as adjacent-cord syringes and a decrease in cord oedema; however, also in this case, the changes did not meet the current criteria for radiological tumour response.

Other studies and combination protocols: Bevacizumab was used also in 5 NF2 patients in the study of Hawasli et al. (202), who tested in a sixth patient another inhibitor of angiogenesis, pazopanib. All the NF2 patients showed benefits from anti-VEGF, while among the other patients, not affected by NF2, two (harbouring recurrent meningiomas) continued to present tumour growth after treatment. Subbiah et al. (185) demonstrated the utility of a combined treatment with Bevacizumab and **Temsirolimus**, an mTOR inhibitor, in two patients, who presented a volumetric response (33%) or a stabilization of the disease.

A combination of bevacizumab and radiation therapy has been proposed in a very recent paper by Zhang et al., who hypothesize a probable dual action on angiogenesis (bevacizumab) and oxygen sensitization (radiotherapy) (203). On the other hand, in another study, Bevacizumab was demonstrated to be effective

in patients previously unsuccessfully treated with gamma knife radiotherapy (204).

Chronic pain: A significant pain relief was reported in a 27-year old patient with severe lumbo-sacral pain due to a spinal schwannoma, treated with a dosage of 5 mg/kg every second week (205); at the same time, her deafness did not improve and VSs did not show any volumetric shrinkage. In a larger retrospective study, five NF2 patients went on to receive infusions of bevacizumab, with four reporting a decrease in subjective pain. All patients that had pain relief had a relapse of pain symptoms when the dose was reduced or infusions stopped (206).

Anti-VEGF in the paediatric age. Results for NF2 children are still limited. Seven children and teenagers affected by NF2 (median age 15 years) were treated by Hochart et al., (207): one showed a tumour regression of more than 20%, two a tumour shrinkage between 5 and 19% and the other four presented a decreased tumour growth or a stabilization. Four patients were eligible for audiometric evaluation, and only one showed a hearing benefit, while the others a stabilization of the disease. Severe adverse events (hypertension and osteitis) were registered in two patients, who had to discontinue the treatment; another patient needed a reduction of the doses due to recurrent episodes of epistaxis, while a female experienced a grade 2 inter-menstrual bleeding.

Efficacy in children was compared to that of adult age in a large multi-centre UK study: in this case-series, six children with NF2 (8 evaluable VSs) had significantly poorer responses to bevacizumab (188).

2nd Chapter

The Italian Network: A study on NF2 clinical presentation in childhood and on bevacizumab treatment for NF2-related tumours

1. The Italian Network

The "Italian Network on Childhood NF2" has been instituted by an agreement among ten 3rd level referral centres, spread in the entire Nation. The units involved were:

1) Unit of Rare Disorders of the Central Nervous System in Paediatric Age, Department of Clinical and Experimental Medicine, University of Catania.
2) Unit of Neuro-Oncology, Scientific Institute for Research, Hospitalisation and Healthcare "Giannina Gaslini", Genova
3) Unit of Neuro-oncology, Department of Paediatrics, Hospital "Meyer", Florence
4) Units of Infantile Neuropsychiatry, Policlinico S. Orsola-Malpighi, University of Bologna
5) Regional Centre for Paediatric Genetics and Rare Disorders, Hospital "Pugliese Ciaccio", University "Magna Graecia", Catanzaro
6) Units of Dermatological Rare Disorders and Neurocutaneous disorders, Policlinico "Umberto I", University "La Sapienza", Rome
7) Unit of Paediatric Neurology, Policlinico "Umberto I", University "La Sapienza", Rome
8) Unit of Oncology unit, Hospital of Macerata
9) Unit of Othorinolaringoiatry, University-Hopital of Padua
10) Unit of Neuro-oncology, Neurological Institute "Carlo Besta", Milan

In each centre a clinician (paediatrician, neurologist or dermatologist) had to collect clinical, laboratory and instrumental data from the patients enrolled. The centre of Catania was the coordinator centre and the Author of the present work, together with his co-tutor, have personally visited and collected data from all the patients involved in the study.

2. Congenital and Childhood NF2 in Italy

The first part of the research project, which involved all the centres of the Italian Network on NF2, was a retrospective analysis on the clinical, radiological and genetic findings of patients affected by congenital (within the first year of life) and infantile (*Wishart-type*, with clinical onset before the adolescence) NF2. It was a retrospective analysis mainly based on patients' clinical records, as well as visits and interviews to patients and/or their parents.

2.1 Patients and Methods

Patients were identified using their initials and a unique identifier number based on the centre involved. Their data included: date of birth, prenatal and neonatal history [weeks of gestations at delivery, modality of delivery, weight, length and head circumference at birth]. Age at symptom onset, as reported above, allowed to distinguish patients with congenital to patients with *Wishart*-type NF2. The main symptoms at onset were reported in the form of an "open-answer" (e.g. headache, visual impairment, abdominal

pain, skin signs), and then grouped in different categories: vision, hearing, neurological symptoms, headache, peripheral neuropathy, cutaneous anomalies. A second part of the questionnaire aimed to evaluate the age at diagnosis, with data drawn from their first clinical and instrumental evaluation, which included hearing assessment, visual functions, assessment of skin anomalies and central nervous system, cranial nerve, spine or peripheral nervous system involvement. The same data were verified in the follow-up visits.

Instrumental examinations included skin Wood's lamp, full ophthalmological examination, audiometry and auditory evoked potentials, and brain and spinal MRI.

Lastly, the genetic assessment and findings of the children (i.e. specific variant found in *NF2* gene, if present) together with data on treatment (i.e. surgery, radiotherapy, anti-neoplastic drugs), as well as their outcomes were reported.

2.2. Statistical Analysis

Data were statistically analysed, where possible, with values of mean and standard deviations, as well as figures on prevalence of symptoms or features. Data found in our patient were compared to those found in literature in a meta-analysis which included 330 paediatric patients, reported in case-series or single case reports **[Tables 3 and 4].**

2.3. Results

Twenty-four patients were enrolled (9 Males, 37.5%). Average age at onset was 5 years: two patients (8.3%) were affected by congenital NF2, while 22 (91.7%) by Wishart-type, prepubertal NF2. Five patients (20.8%) had a parent affected by NF2, while 19 (79.2%) had any relative affected. At onset, symptoms involved the central nervous system and/or cranial nerves in 15 cases (62.5%), skin in 13 (54.2%) with skin tumours or cafè-au-lait spots, peripheral nervous system, mainly neuropathy, in 9 (37.5%), hearing loss in 4 (16.7%), but only one patient (4.2%) presented radiological findings of Vestibular Schwannomas. Three (12.5%) presented visual impairment, 2 (8.3%) abdominal masses and only one (4.2%) a spinal ependymoma [**Table 3**].

During the first ten years of the disease, all patients have presented cafè-au-lait spots, and the average of spots per patient was 2.12; twenty-one (87.5%) presented also skin tumours. In the same period, the hallmark of NF2 (bilateral vestibular schwannomas) has been found in 17 patients (70,8%), with 10 (41.7%) presenting also hearing impairment. Problems in vision were more common (11 patients, 45.8%), with cataract present in 8 patients (33.3%). None of the patients have presented retinal hamartomas.

Headache has been never reported, and seizures only in three patients (12.5%). Cranial nerve tumours other than VSs have been noticed in 4 patients (16.7%), but symptoms of involvement of cranial nerves (e.g. amblyopia, strabismus) present in 11 patients (45.8%).

A high percentage of patients have presented spinal cord tumours (20 patient, 83.3%), while meningiomas have been harboured by 14 patients (58.3%). Thirteen (54.2%) patients experienced some form of peripheral neuropathy, and in two cases (8.3%) an abdominal schwannoma was found.

Outcomes were good (i.e. absence of invalidating symptomatology, hearing loss, visual impairment) in 11 patients (45.8%); ten (41.7%) presented some form of hearing impairment. Other symptoms presented in the later ages were paresis (3 patients, 12.5%), vertigo, diplegia (2 patients each, 8.3%). One patient (4.3%) presented blindness. Three patients (12.5%) presented "poor outcome" (i.e. severe symptoms or chronic pain), and 2 (8.3%) deceased within 10 years from the diagnosis.

3. Bevacizumab for NF2-related tumours

3.1 Patients and Methods

Twenty-five patients affected by NF2 and treated with bevacizumab were enrolled in a prospective study. Centres #5 and #7 had patients affected by paediatric NF2 but no one was treated with bevacizumab. Patients were diagnosed as affected by NF2 according to the modified Manchester Criteria, which consisted of 1) presence of bilateral VSs; 2) occurrence of other tumours typically related to NF2 (intracranial meningiomas, other cranial nerves schwannomas, spinal ependymomas); 3) genetic confirmation of the disease; 4) at least on first-degree parent affected by NF2 in absence of VSs or other NF2-

related tumours or doubtful genetic tests. Patients were enrolled if affected by congenital NF2, *Wishart-type* NF2, *Gardner-type* NF2 as follow:

- Congenital NF2 → Occurrence of bilateral VSs within the first year of life.
- *Wishart-type* NF2 → Onset of NF2 after the first year of life and before 12 years of age, with severe pre-pubertal involvement of the central nervous system.
- *Gardner-type* NF2 → Later onset NF2

Patients could have performed an attempt of surgery care in one or more NF2-related tumours (VSs, meningiomas), as well as a previous radiotherapy or gamma-knife treatment. Exclusion criteria was a concomitant use of other neoplastic drugs.

Before the beginning of the treatment, all patients underwent to a base-line brain and spinal MRI, with thin cut (3 mm) and with T1, T2, FLAIR and contrast-enhanced sequences; base-line hearing assessment consisting in pure-tone average (PTA), speech recognition threshold (SRT) and speech discrimination score (SDS), even if, for study purposes, hearing threshold at 20, 400, 4000 Hz were assessed.

Before the study, all patients underwent to a full panel of laboratory examinations including full blood count, hepatic and kidney functions, electrolytes, coagulation factors, infectious disease screening, RCP and ESR evaluation.

The study aimed to collect data on volumetric shrinkage of NF2-related tumours: VSs, other cranial nerves schwannomas, meningiomas, spinal ependymomas size was evaluated on the basis of three-dimensional volumetric MRI, mainly because linear diameter measures underestimate the volumetric growth or reduction of brain tumours. Hearing assessment was another aim of the study, as in many study a volume shrinkage in the MRI has not corresponded to a hearing improvement of the patients.

To assess the efficacy of treatment in tumour reduction and functional improvement, in all patients, we calculated the growth-rate of tumours (in percentage, per year) presented in the three years preceding the treatment, and compared it with the growth rate during treatment. The same was calculated for hearing decay/improvement (also in this case in percentage), in the three years before bevacizumab and during the treatment.

For patients affected by other neurological deficits (i.e. neuropathy, vision loss), a serial evaluation of their deficit was performed either by clinical visits, or instrumental assessment (visual potentials, electromyography, nerve conduction velocity).

Patients underwent to laboratory investigation every three months, MRI, hearing and other instrumental assessment (including, when necessary ophthalmoscopy, nerve conduction velocity and electromyography), every 6 months. Follow-up included also general neurologic evaluation and neuropsychological assessment.

Approval of the local Ethical Committee for compassionate treatment with bevacizumab was obtained according to the Italian Off Label Drugs legislation (law 648/1996) and the Decree of the Ministry of Health on the Therapeutic Use of Medicinal Products Subject to Clinical Research (*8 May 2003*). All patients (or their caregivers, if younger then 18 years of age) provided written informed consent for treatment. Bevacizumab was given at a standard dosage of 5 mg/kg every two weeks. For all the duration of the treatment patients have been asked to record a clinical diary on the side-effects presented. Toxicity data were collected during routine clinic visits and were scored according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 4.0 (208). Treatment had to be suspended if one of the following conditions occurred: 1) grade 3-4 allergic reactions (i.e. anaphylaxis, respiratory arrest, dyspnoea, massive oedema); 2) grade 2-4 hemorragic complications; 3) grade 3-4 infectious diseases; 4) 3D tumour progression of > 20% (no response to treatment).

3.1.1 Hearing response assessment

Hearing response was determined by an audiology test (pure tone thresholds and word recognition scores) every 6 months. Auditory results improved by more than 20% were defined as major, ranging from 5% to 19% as "clinically appreciable" (minor response) and less than 5% to -5% as "stable", and less then -5% as non-satisfactory (no response). However, for the purposes of the

present study and in consideration of a statistical analysis of pure tone audiometry, we compared values (in decibel) of threshold at frequencies of 40, 400 and 4000 Hz, calculating the average changes of audiometry in single patients and in the total of patients.

3.1.2 Magnetic resonance evaluation

A tumour radiologic response was defined as "minor" when the volume decreased between 5 and 20 %, and "major" when the decrease was greater than 20 %, as accepted criteria for NF2 VSs. If the difference ranged from -5 to +5% the diseases was defined as "stable". An increased volume comprised between 5 and 20% was defined as "progressive disease", while an increment of more than 20% was defined as "non-response".

Vestibular schwannomas: Bilateral vestibular schwannomas are the hallmark of NF2 and the main objective of the present study was to evaluate the action of bevacizumab against these tumours. Bilateral VSs was not a mandatory condition, since many patients could have been treated by surgery previously, with total removal of one of the tumours. In doubt cases, to investigate whether tumour shrinkage was related, in part, to a decrease in intratumoral vasogenic oedema, a DWI-MRI was performed: this technique determines the mean apparent diffusion coefficient, a measure of the magnitude of diffusion of water molecules within tissue and a reliable marker of oedema within vestibular schwannomas.

Meningiomas: In case of multiple meningiomas, the volume of the two largest tumours was taken in consideration. Total intracranial tumour volume was defined as the sum of the volume of these two intracranial meningiomas. We used MRIs performed prior the start of bevacizumab therapy to determine pre-treatment meningioma volume. All tumour contours were taken using T1 post-contrast axial MRI scans. For tumours with indistinct boundaries, a skilled neuroradiologist reviewed axial, sagittal, and coronal slices of pre- and postcontrast T1-weighted, T2-weighted, diffusion-weighted images, FLAIR, susceptibility, FIESTA images, and CT scans when available.

Spinal ependymomas: In case of multiple ependymomas, the volume of the two largest tumours was taken in consideration, using head and spine MRIs performed with intravenous gadolinium contrast; ependymoma size (in mm) was measured using T2-weighted images. As in other studies (200), an exact volumetric assessment of ependymomas was impossible to perform due to the inhomogeneous appearance and cystic tumour formation and the volume was therefore calculated using the geometric formula of the volume of the sphere $(4/3*r^3*\pi)$, using as the radius half of the maximum diameter of the tumours.

3.2 Statistical Analysis

Statistical evaluations were performed using the statistical program Sata. We used correlation for analyses of mean tumour shrinkage and calculated the

growth-rate of tumour, as well as the hearing decay, before and after treatment. A p-value minor than 0.05 was considered as statistically significant.

3.3 Results

Twenty-five patients were included, harbouring 40 VSs, 15 meningiomas and 11 spinal ependymomas. Median age at enrolment ranged from 10 to 49 years (average 21.36 years). Treatment duration ranged between 1 and 5 years. The average treatment duration was 4 years (median 4 years). Twenty patients are still treated with bevacizumab at the end of the present study [**Table 5**].

For VSs [**Table 6**], after one year, treatment was effective in 18/25 of patients (72%) affected by VSs, who showed a reduced or stable volume for the entire duration of bevacizumab trial. In particular, 4% of patients showed a volume decrease of > 20% of the VSs; 28% of patients presented a "minor response" (volume shrinkage between 5 and 19% of the initial volume), and 40% a stable disease. Disease progression, defined as more then 5% in comparison to the original mass, was observed in 7/25 of patients (28%). In patients presenting severe side effects or severe unrelated conditions, as well as a tumour growth of more than 20%, treatment was ceased: this happened in three patients (12%). One of these, two years after the discontinuation of the drug, died because of massive tumour enlargement in head and left pelvis and a concurrent severe infectious disease (pneumonia), apparently not related to bevacizumab.

After five years, 9% presented a volume shrinkage >20%, 36.3% a "minor response", 27.3 % a stable disease, 13.6% a tumour progression, and 13.6% a treatment failure (tumour growth >20% and/or drug suspended). In total, Bevacizumab treatment was related to tumour decrease or stable disease in 16/22 (72,7%) of the patients who were still treated after 5 years (64% of the total patients enrolled in the study).

Considering all the patients, the average tumours' growth at 1 year was 1.87% and 0,67% at 5 years, compared to a mean of 27% in the years before the treatment.

Auditory assessment [Table 7] was partly related with the effects on the volume of tumours, with audiometry results showing objective improvement in the patients with a tumour volume shrinkage. The mean volume improvement in all the patients, for both years, was 0,42% after 1 year, and 0,61% after 5 years. In the years before the treatment, hearing decay had been 15.3% per year. However, after 1 year, patients who presented major hearing improvement were 4%, minor improvement 16%, stable disease 60%, progressive disease 20%; after 5 years (on 22 evaluable patients), major response was present in 4.5%, minor response in 36.4%, stable disease in 27.3%, progressive disease in 31.8%. Considering the total of 22 patients enrolled in the study who had continued the treatment for 5 years, 68.1% presented a stable or improved hearing assessment (60% of the 25 patients enrolled at the beginning of the protocol).

Efficacy of the treatment in patients affected by meningiomas (10, 40% of the total) was comparable with that obtained in VSs [**Table 8**]. Eight patients were treated for 5 years, and at the end of the follow-up, we observed an overall shrinkage of tumours > 20% in 12.5 % of patients, a minor response in 25 % stabilization of the growth in 37.5% % and a tumour progression in 25.5 %. To note, two additional patients could be also evaluated at a 1-year follow-up, both showing a tumour growth arrest: one of them, at the same time, showed a severe outcome regarding his vestibular schwannomas, and had to interrupt the treatment. The mean volume reduction observed in the patients was 4.17% at 1 year and 1.86% at 5 years; before treatment tumour growth was on average 17.21% per year. The tumour shrinkage was not correlated with tumour size prior to treatment and volume shrinkage of their vestibular schwannomas.

For the seven patients (28%) affected by spinal ependymomas [**Table 9**], at 1 year, a stabilization of the disease was observed in 57,1 % of patients, a minor response in 28.6% of patient (none of them presented a tumour volume shrinkage > 20%). One patient (14,3 %) presented a tumour progression. At the maximum follow-up of 4-5 years, results on 5 patients were available: two (40%) presented a stable disease, while 3 (60%) a tumour progression (one of them a tumour growth > 20% from the baseline).

Average tumour growth was -0.4% after 1 year and +7.28% after 5 years: in this last case, there was no statistically significant difference with the tumour growth before bevacizumab treatment (17.3% per year).

Treatment was well tolerated. Side effects were observed in 5/25 patients (20%), and consisted in nausea (2 patients), fever (1), massive inter-mensual bleeding (1), proteinuria (1) and piastrinopeinia (1). In some cases, treatment was discontinued for a period spanning 3 weeks to 1 month, and after normalization of the hematic parameters (e.g. platelet count, protein levels), the treatment was re-initiated without any other side effect in all the patients, with the exception of one of them presenting three different episodes of proteinuria.

3.4 The case of patient #003: first report of a dramatic recovery of vision after bevacizumab

He was a 12-year-old Italian boy, born pre-term (gestational week 36) by complicated delivery (torsion of the umbilical cord) after normal pregnancy. He presented congenital right microphthalmia, and at age 3 months, an eye examination revealed right lens opacity associated with pigment retinal hyperplasia.

At the age of 4 months, a magnetic resonance imaging (MRI) showed left colpocephaly and bilateral high-signal (partially calcified) lesions in the posterior periventricular region. In addition, the brain study revealed small, bilateral VSs. Auditory evoked potentials yielded normal findings and a first DNA analysis for the NF2 gene yielded negative results.

At age 18 months, his parents had noticed a decrease in visual acuity in the left eye. In addition, he had developed multiple raised lesions in the hands

and leg and additional plaques in the face and trunk. Visual acuity was 1/50 in the right eye and 3/10 in the left eye. Full ophthalmologic examination revealed a pale optic disc in the left eye. A histological study of two skin lesions taken from one hand and leg confirmed these as NF2 plaques. Again, auditory evoked potentials yielded normal results. A new brain and spinal MRI study confirmed the previous findings with VSs of unmodified size, and revealed **left optic nerve meningioma**, with no other lesion in the brain and spinal cord. A new sanger sequencing of *NF2* gene revealed a mutation in codon 94 (c.281_282 ins CCTT) of exon 3 only in the proband.

In the course of the years, he manifested an increase in the number of NF2 plaques, especially in the face, hands and feet, which tend to disappear after the age of 10 years. One year later, he presented progressive hearing loss, sudden gait unbalance and episodic vomiting. There was bilateral hearing dysfunction (word recognition scores were as low as 7% in the left ear and 34% in the right ear) with abnormal auditory evoked potentials. Cerebellar ataxia was also present. The brain and spinal MRI showed marked progression of the VSs with mass effect and displacement of the brainstem and mild increase in the size of the optic nerve meningioma **[Figure 8]**. The child underwent surgery with left vestibular schwannoma excision and clinical improvement.

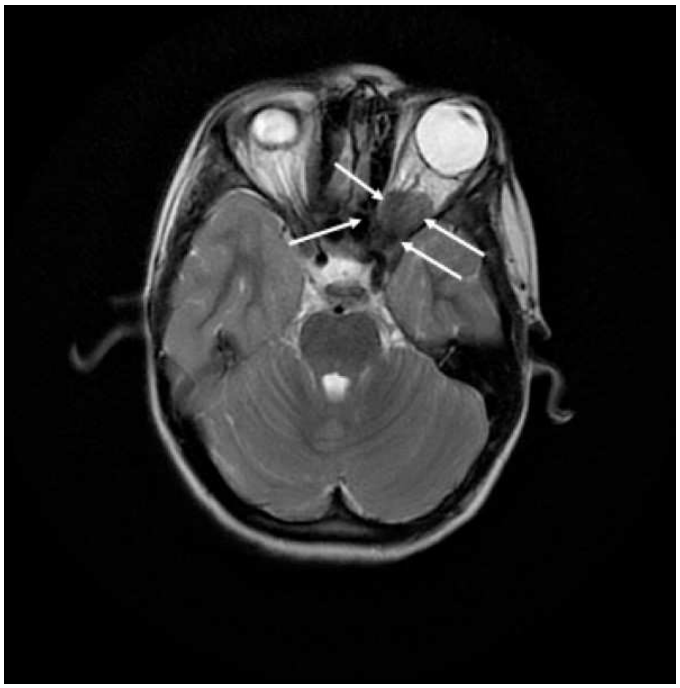

Figure 8. Axial, T2-weighted MRI of the patient, at 10 years of age. A left optic meningioma (white arrows) compresses the optic nerve.

Pharmacological treatment with bevacizumab [5 mg/kg intravenously every 15 days] has been started since mid-September 2012 because of a decrease in hearing parameters: after administration of seven intravenous doses (i.e., after 75 days of therapy), a clinical improvement was recorded, consisting in disappearance of cerebellar signs and amelioration of hearing and visual function. Specifically, the cerebellar signs started to disappear two days after the initiation of therapy and the child's word recognition scores increased from 9% to 67%: the improvement in word recognition began three days after the initiation of treatment and continued to improve as long as the 75th day of therapy. The patient visual perceptions also improved, and this was documented by the evaluation of the evoked visual potential **[Figure 9]**, in which the

amplitude of P3 wave increased, while the latency remained unchanged, thus showing an increase in number of conducting fibres, even if the conduction of the impulse in the optic pathways remained delayed.

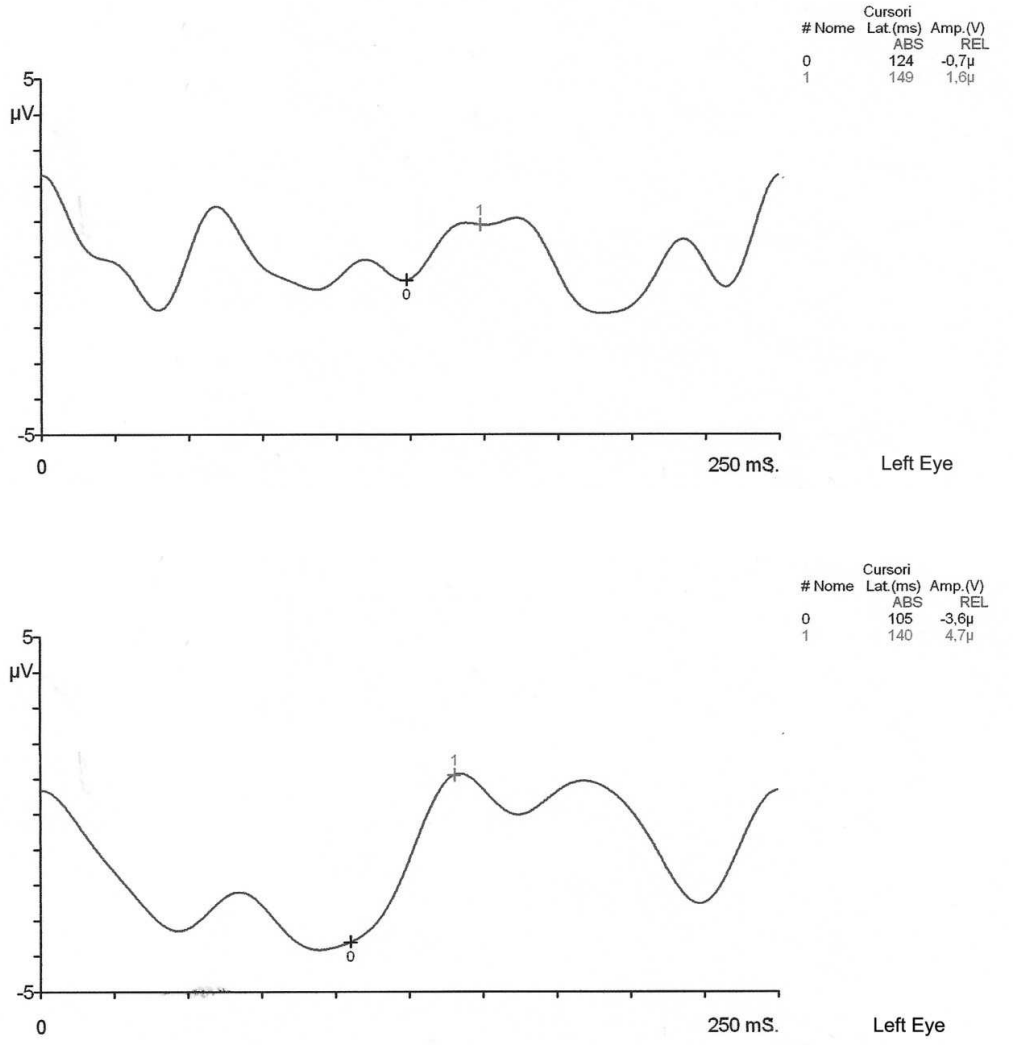

Figure 9. Visual Evoked Potential of the patient performed before the treatment (upper image) and after 5 years. The amplitude of the P3 wave increased, thus demonstrating an increase in the number of the conducting fibres

3rd Chapter

Discussion

The present project was a multi-centre, national study on NF2 which can be divided in two different parts. One aimed to evaluate the clinical and instrumental data of patients affected by the paediatric forms of NF2 (congenital, pre-pubertal), trying to find a specific or more frequent sign or symptom, and then comparing these data with those reported for the same patients in the literature. Twenty-four patients were enrolled, in one of the largest series of patients reported in the last twenty years.

The second part of the study aimed to evaluate in NF2 patients of all the ages, the effect of the treatment with bevacizumab on all the tumours related to the disease, evaluating all the possible clinical and instrumental findings of the patients.

1. Clinical presentation of NF2 in Childhood

Several studies have highlighted the difference in presentation between Gardner and Wishart-type NF2, from the onset of the disease, to the natural history. and evolution of the disease (26, 32, 135, 147).

1.1 Symptoms at onset

Commonly, the first signs in adulthood are those related to bilateral VSs, which include hearing loss, tinnitus, or balance dysfunction, and their evidence at MRI as bilateral lesions, allow a diagnose of NF2 (209, 210). NF2 children have a different clinical presentation: VSs are reported in a minority of cases, and, a

part from genetic finding of NF2 mutation, specific diagnostic criteria are absent (137). Skin tumours, posterior capsular cataracts, or neurological signs secondary to schwannomas in other-than-vestibular nerve(s), and/or brainstem or spinal cord compression are more common and may be present long before dysfunction of cranial nerve VIII (133): in the present series only one patients (4%) had a VS as his first sing of the disease, not different from the incidence found in the 330 cases reviewed (8%) (211-213). However, haring concerns were the presenting sign in 11% of patients reported in literature and in 16% of our series, thus underlying the probable occurrence of (some) auditory dysfunction in cases of very small, not already noticeable, tumours.

Generally, signs related to central and peripheral nervous system tumours, excluding the VSs, were the most frequent reason of medical visit for our patients. In contradistinction to a previous report (135), but in line with other studies (136, 138), we found no child who presented with an isolated schwannoma or meningioma, and subsequently all (even the youngest patients) met the classical NF2 criteria of "multiple cranial tumours or bilateral VSs"

In childhood, the involvement of other cranial nerves with symptoms consisting in amblyopia, strabismus, facial palsies as well as other symptoms including retinal hamartomas, epiretinal membranes, was frequent. Involvment of cranial nerves or central nervous system was the main symptom of 64.2% of our patients, and 11 (45.8%) patients were refereed to physician for this kind of

signs. In six children was given a diagnosis of either idiopathic strabismus or amblyopia before the development of other neurological symptoms that prompted the diagnosis of NF2. Even though strabismus and amblyopia have been regarded as non-specific, we recorded both signs in children who later on in their NF2 course were shown at neuroimaging to have schwannomas of the third, fourth, sixth, and seventh cranial nerves or tumours in the skull base or in the brainstem, in line with the consideration that an acute-onset strabismus, especially in childhood, has always to be considered as a red-flag for the presence of a brain tumour. Revising the literature, we found that involvement of cranial nerves or more generally of brain was present in 23.5%, with exclusively cranial nerve in 12% of patients. To note, Evans et al. found six out of 61 children presenting with unilateral facial palsy not associated with meningiomas or schwannomas of cranial nerve and this was particularly frequent in the fully detailed patients below 10 years of age (5 out of 30, 16.6%) (135)

Brainstem symptoms, in adult age, have been related to the compression of VSs in the cerebellar-pontine angle, and the occurrence of related symptomatology as a possible sign of VSs (214). In the present cases, however, brainstem signs were much more related to primitive brainstem anomalies and not to VSs, and most of patients who developed brainstem tumours as a presenting sign at the time of first neuroimaging investigation had not developed any vestibular schwannoma; furthermore, we also recorded cases

who had lower cranial mononeuropathy.

We also found an high prevalence of spinal cord tumours, with a frequency of 25% in the present patients (in one patient – 4.2% - as the only sign with a spinal and abdominal mass). In literature, such tumours were present only in 3.5% of patients, at onset (213, 215).

These data well relate to the presence of neuropathy, including with this term a lot of manifestations as paraplegia, hemiplegia, diplegia, monoplegia, paresis (paraparesis, hemiparesis, monolateral paresis) and sensitive manifestations as paraesthesia: in 9 of our patients, peripheral nervous systems signs represented more than 37.5 % of the initial manifestation of NF2. Focal muscle wasting was another sign related to schwannomas of the dorsal nerve roots of the cord or in a large nerve plexus (216). We found a minor incidence of symptoms related to peripheral nervous systems, in literature, with 6.5% of patients presenting neuropathy as first manifestation (138, 217-219).

In rare cases, NF2 may manifest with (few) cafè-au-lait spots and associated to peripheral nerve tumours, thus resembling NF1 or schwannomatosis (and being misdiagnosed). Diagnosis is usually revised when other other tumours become symptomatic or other NF2 features manifest (146). In nine cases (37,5%) of the present series, patients were initially referred for cafè-au-lait spots, and (erroneously) reassured of not having NF because of the limited number of macules (average 2,12 macules per patient). A deeper

analysis of their clinical history, allowed us to find other findings which could be related to NF2: cataract in one patients, neuropathy/cranial nerve system involvement in 5. Remarkably, in all these 8 cases the cafè-au-lait spots had irregular margins and with a lighter colour compared to NF1 patients, irrespective of age: however, it is well known, from histology reports, that there is a lower numbers of melanin macroglobules in NF2 than NF1 cafè-au-lait spots (144). Other cutaneous findings (i.e. skin tumours) were noticed in 7 patients (29%), and, overall, skin manifestations were present in 54.2% of the patients, much more than reported in literature, where cafè-au-lait spot and skin tumours present in 4% of patients only (137, 147).

Regarding the age, onset before one year of age – the so-called Congenital NF2 – was present in two patients of our series and both with a very severe clinical presentation consisting in eye symptoms, VSs, cortical dysplasia. Similar presentation, in patients of this age range, was already reported (135, 136, 138).

1.2 Natural history

In the course of the disease, clinical manifestations may vary over the time, involving organs and district spared in the initial phases of the disease.

Skin manifestations. Even if skin manifestations are more typically related to NF1, cafè-au-lait spots and skin tumours (i.e. NF2-plaques) may be found also

in NF2, but in many cases, it is difficult to distinguish some of the cutaneous and nodular schwannomas from neurofibromas. However, NF2-plaques, which are wide schwannomas of the thin nerve of the upper layers of the skin, have distinctive appearance as discrete, well- circumscribed, slightly raised pigmented cutaneous lesions often containing excess hair and usually less than 2 cm in diameter (32). All our patients developed, during the course of their disease, cafè-au-lait spots on their bodies (average of 2.12 macules per patient) and 21 patients (87.4%) presented skin tumours. These lesions tended to grow in number and size with age. Previous reports have showed that these abnormalities are more common in patients with earlier onset and more severe disease and become more visible with age (138) and plaques are typically localized in the trunk (135); this last aspect is slightly different from what we observed in the present patients, in whom the most common location were upper and lower limbs (including hands and feet). Prevalence of cafè-au-lait spots and skin tumours was another different aspect between our patients and literature data, where only 13.3% showed cafè-au-lait spots and 9.4% skin tumours (136, 137, 147, 217, 219); higher figures were found by Nunes et al., who showed in their study that 25% of all patients had skin tumours (138). However, it is interesting the strict correlation between skin tumours and cranial nerve tumours: when these last are absent, NF2-plaques are absent with a ratio of 30:1; in addition, if cranial nerve tumours are present, skin tumours are present with a ratio of 1:3.

Ocular manifestations. As seen above, the eye may be one of the first organ affected by NF2, with cataract, lens opacities, retinal hamartomas and epiretinal membranes. In our patients, visual impairment has been noticed in 11 patients (45.8%): 8 of them (33.3%) presented cataract, the most common types being posterior subcapsular or cortical. In all the largest NF2 series, lens opacities occour without symptoms, but when they are large they can affect visual acuity in approximately 20 % of NF2 patients. In childhood, NF2 lens opacities are often detected by chance very early in life but often thought to be sporadic: children are then discharged with a follow-up visit planned in 3-6 months(32). However, the association with hearing loss or neurological signs should alert for the risk of NF2. Moreover, even if NF2 is a rare cause of congenital cataract, all individuals at risk for NF2 should be screened for its presence in childhood; in the same time, a child with congenital cataract as an isolated feature should be reviewed and assessed for other features of NF2 (220). A further 24 % of our cases had retinal hamartomas.

In literature patients, the incidence of generic visual impairment is of 12.42%, while cataract was present in almost the double of patients (21.21%). Retinal hamartomas were rare, with an overall incidence of 4%(136, 221) . Notably, Evans et al. recorded lower overall figures (3%) for cataract in their NF2 children (4 patients), finding that, like for adult age, this is not a frequent sign of the disease, with the most frequent types being the bilateral or posterior capsular ones (135) (Evans et al.). In the literature review, we found also other

severe conditions associated to NF2, like vitreoretinal degeneration with retinal detachment in two male cases: one had a retinal detachment at age 5 years, the second, age 15 years, developed retinal tears and early detachment (129). Many Authors reported that pigment epithelial retinal hamartomas, beside other congenital changes of the ocular fundus, may help to establish the diagnosis early in the course of the disease, even in absence of vestibular schwannomas (211, 217)

Vestibular Schwannomas and hearing loss. Otolaryngologic manifestations represent the most specific sign of the NF2 and hearing loss and tinnitus are common finding in these patients. From the present case series and from literature review, however, it appears that in childhood these may not be present in all the patients, and rarely are the first symptom of the disease.

In our patients, we observed an incidence of 17 cases (70.8%), with 10 (41.7%) showing progressive hearing loss. However, it should be noticed that some otolaryngology symptoms can be caused, as well, by intracranial meningiomas. NF2-related bilateral VSs tend, however, to manifest earlier than the monolateral (non NF2-related), and present a larger size, a multilubulated aspect at microscopy, as well as in important component of edema in their tissues and in the surrounding ones. In literature, the presence of bilateral vestibular schwannomas was found in 23.27%, isolated in 4%; hearing loss was present in 22.42% of them. The presence of early schwannomas can be

overlooked by normal MRI, and thin cut and gadolinium enhancement, even in the first years of life, may be necessary, in patients with positive familiar history (222). Moreover, VSs may be present for years before causing some hearing loss: in the experience of Mautner, on six patients with VSs, only one presented with hearing loss (136). More recently, Choi et al. confirmed the previous data, showing that 68% of their patients (17 out of 25) has bilateral vestibular schwannomas, but only 20% complain some hearing discomfort. These last patients were mainly affected by rapid-growth tumours (213). Lower figures were found by Bonne et al., who found symptomatic bilateral vestibular schwannomas in only a third of their patients, confirming the importance of an early diagnosis, essential for performing an acoustic nerve-sparing surgery with following cochlear implantation (219).

Other cranial nerve involvement. Cranial nerve symptoms represent another important manifestation of NF2, with higher prevalence in children than in adults. In our experience, 11 patients (45.8%) had signs or symptoms related to an involvement of cranial nerves different than VIIIs: the main symptoms were those related to cranial nerves III, IV and VI (strabismus, amblyopia and ptosis), VII (facial palsy), IX and X (hipcup and bitonal voice) and XII (tongue atrophy). Beside the direct role of tumours, many patiants can present with symptoms even without a detectable mass and it is thought that the mutation of merlin can cause a dysfunction of integrity of Schwann cells, producing an irregular transmission of the signal and developing the neuropathy; another

explanation could be the development of small , microscopic schwannomas in peripheral nerves which are not detectable with the MRI (223). However, in the same literature, we found a minor incidence of symptoms related to cranial nerve involvement than tumours (16.6% vs. 23.9%): like for VSs, not all the tumours cause symptoms and a primitive neuropathy could be the cause of the symptoms (32, 136).

Tumours of the central nervous system. The morbidity and mortality caused by NF2 is largely due to intracranial and spinal tumours (32), and a fulminant clinical course, albeit rare, was related to the CNS tumour burden in previous series of early-onset NF2 cases who exhibited progressive deterioration with loss of hearing, ambulation, and sight along with chronic pain (137, 138, 224). In our cases, meningiomas have been reported to occur in 58.3% with intracranial involvement, other-than intracranial meningiomas seen in 33% of patients; headache and seizures were uncommon symptoms. Intracranial calcifications on CT scans not due to tumours and somewhat similar to those seen in tuberous sclerosis complex have been reported in a number of NF2 patients (225), as well in two of our patients who had in addition either periventricular or cortical high signal lesions resembling cortical dysplasia. The latter finding has been reported in association to NF2 and attributed either to meningoangiomatosis or to hamartomatous lesions. Of interest, all the high signal lesions recorded in our series were asymptomatic as reported in four of 11 NF2 cases who had meningoangiomatosis. In the review of literature, data

confirm what we have found: 20.6% had cranial meningiomas, other-than intracranial meningiomas were present in 9%. Behavior or meningiomas in children affected by NF2 is often severe (especially when associated to truncating non-sense or frameshift mutation), and especially in childhood, NF2 is one of the most frequent causes of meningiomas occurrence (10-18% of all children who develop meningiomas has a subsequent diagnosis of NF2) (113, 226). Bosch et al. have found that childhood onset NF2 is always associated with at least 2 cranial tumours, with an additional development of other 2 cranial tumours after ten years of follow-up (227).

Cranial tumours were more frequent than spinal at onset, but during the follow-up, 20 of our patients (83.3%) developed spinal cord tumours (especially schwannomas), which were diagnosed by MRI. These tumours often remained asymptomatic as reported in previous childhood series (136, 138). However, they represent an important finding and can help to diagnose NF2, because they are typical and very rare as sporadic tumours. In literature 40,91% presented spinal cord tumours, and in particular many authors emphasized the association between spinal cord tumours (especially schwannomas) and NF2, and in particular they allow to differentiate NF1 (in which these are absent) to NF2 (in which they could be present also when VSs are absent). Given the fact that, especially in the first phases, both spinal meningiomas and ependymomas are asymptomatic, a cautious behavior is important, limiting the surgical treatment to the patients who develop symptoms (228).

Neuropathy. 54.2 % of our cases show this manifestation during their disease, presenting with different symptoms, with the possibility to involve one or more arms, sensitive or motor neurons. A minor figure was found in literature, where only 9% of patients had neuropathy. The physiopathologic mechanism is not completely understood: in some individuals, it is due to spinal cord tumours, others have the subjective manifestations (recorded also by neurophysiological studies such as electromyography and electroneurography) in absence of tumours. The same mechanisms involved in cranial nerve neuropathy can be the cause of peripheral neuropathy: the loss integrity of Schwann cells would cause an irregular transmission of the neurological signal and the symptoms, or subtle, undetectable micro-schwannomas may be the real cause (223). It is important to underline that NF2-associated neuropathy may overlap the symptoms of a poliomyelitis: in this case, neurophysiological studies only can distinguish the diseases (229). In the same study that found that facial nerve palsy may not be related only with schwannomas but also idiopathic, it was found that peripheral neuropathy is frequent in pediatric patients, also at the onset of the disease (135). Our studies and those found in the literature show a strong association (positive or negative) of neuropathy with other complications of NF2. When it is absent, the patients have a higher risk (12:1) of developing cranial nerve symptoms, seizures, intra-cranial meningiomas or there is no familiarity for NF2. On the contrary, if seizures, cranial nerve involvement are absent (and the patient has a familiarity for NF2), neuropathy is highly likely to

be present, with a ratio of 1:8. This may be a casual association or be due to the fact that patients presenting with intracranial symptoms and seizures are very unlikely to refer neuropathy as their main symptom.

Treatment/Outcomes. The tumour load in this as in other childhood NF2 series was extensive and involved the skin, brain, and spine. Resection of dermal tumours because of cosmetic burden, as recorded in our series, is performed in few NF2 cases (138). This is in line with the patterns of growth of peripheral schwannomas which rarely are disfiguring at these young ages (136). Interestingly, resection of typical NF2-plaques is frequently asked because of diagnostic reasons (138), as happened also in five of our patients before the referral to our institutions: however, in these cases, we performed a clinical diagnosis without biopsy analysis. Twenty-one of 24 patients imaged had one or more extradural masses compatible with schwannomas or meningiomas: despite this extensive load of spinal nerve sheath tumours, only in four cases these tumours compressed the cord itself and required surgical removal because of progressive symptomatology: resection was successful in all without the need of repeated neurosurgical intervention as previously reported (136). Three patients underwent complete resection of intramedullary tumours: histology showed grade 2 ependymomas in all without regrowth. Conversely, pontine tumours (usually, ependymomas and astrocytomas) most often needed repeated partial resection with severe sequelae, mainly because the location and the harmful consequences of extensive resections. According to our experience, the

tumour burden in this location was harmful and changed the course of the disease. In general, the brainstem location of tumours in NF2 is associated with higher grade histology, progressive course and often a fatal outcome: two out of six cases operated deceased because of postsurgical sequelae and complications. The decision to operate early on vestibular schwannomas or wait until symptoms or complete deafness ensue is difficult: three of our patients were scheduled for early vestibular schwannomas resection despite mild or no hearing impairment. None of the present cases underwent either surgery for vestibular schwannomas or radiation therapy for progressive intracranial meningiomas or vestibular schwannomas. Stereotactic radiosurgery has been offered as an alternative to surgery in some selected patients as it can provide good short-term tumour control, but one must take into consideration that radiation exposure may induce, accelerate, or transform tumours in NF2 (105). Some patients underwent also to bevacizumab therapy, whose results are discussed in the next section.

Overall, early death (<25 years) occurred in 12.5% of the present patients and with a higher figure (25%) in the literature.

Genotype-phenotype correlation. Molecular genetic analysis carried in these children or in their affected relatives revealed some typical truncating mutations in all the five familial cases and in two of 10 sporadic cases analyzed. Other alterations were seen throughout the NF2 gene and involved nonsense

mutations, frameshift deletion, and splice site alterations. In our patients, a direct correlation genotype/phenotype was not established, given the lack of data. Two adolescent patients presented in-frame mutations detected, with localized schwannomas born by an affected NF2 mother who harbored the same mutation. Very interesting was the high-rate of non-detection (8 out of 15 cases) in sporadic cases. Several studies have analyzed the causes of this, showing that most undetected mutations are certainly due to somatic mosaicism; however, we were not able, in all the cases, to analyze specimens surgically removed in these patients and thus we could not confirm or disclose this assumption. Other reasons could include mutations in intronic regions, promotor mutations, or large multiexonic deletions.

2. Bevacizumab treatment for NF2

In 8 of the 10 centres involved in the study, treatment with bevacizumab was performed on NF2 patients harboring vestibular schwannomas (in some cases in subjects previously surgically treated for one of the two tumours): primary aim of the study was to evaluate the efficacy of this treatment in the shrinkage of VSs and the variation of hearing scores (pure tone audiometry, word recognition score). The secondary aim was the evaluation of the effect of this treatment in two other tumours affecting NF2 patients: meningiomas and spinal cord ependymomas.

In the previous reports of the literature, a significant outcome in VSs volume reduction has been found in all the different reports (150). When a tumour reduction > 20% was not reached, a substantial arrest of their growth has been obtained, with only a few patients presenting – during the treatment – an increase of tumour dimensions. Treatment efficacy has not been proved for meningiomas (in part) and for spinal ependymomas (for almost all the patients). It should be underlined that ependymomas could be considered, for their evolution, growth and infiltration, as a classical "malignant" tumor, while meningiomas and VSs can be considered as "benign": their (threatening) effects are mainly based on their location (cerebellar-pontine angle, nerve sheath, meninges), with a "mass-effect" over fundamental and critical areas of the brain, such the brainstem. Both meningiomas and VSs do not metastasizes nor infiltrate surrounding tissues (ref).

In our patients, the efficacy of the treatment was remarkable for VSs, with a high percentage of patients (72.7%) showing a tumour growth arrest, minor tumour shrinkage or tumour size reduction >20%, and 27.3% a tumour progression. However, if the total of 25 patients initially enrolled in the study is considered, a lower figure of patients (64%) presented stabilization or improvement of the disease. Considering the fact, as stated above, that VSs may be strictly considered as "benign" tumours, it is not surprising that many of these patients may have presented a substantial tumor-growth arrest. For this reason, the main clinical outcome to evaluate, in patients affected by bilateral

VSs, can be considered the comparison between tumour growth rate before and after treatment, and the clinical progression of their hearing scores. The patients recruited, presented, before enrollment, an average tumour progression of 21.49% per year, and the majority of them (72%) already presented some concerns in hearing (average maximum threshold lower than 40 dB) and hearing decay had been 15.3% per year, on average. The average VSs growth after bevacizumab treatment was 1.87% after one year and 0.36% after five years, thus showing a substantial "stabilization" effect of this treatment for these tumours. In the same time, hearing improvement was 0.41% at 1 year and 0.61% after 5 years.

The clinical improvement / stabilization of patients' hearing is the most important result of this treatment, as previous surgical options or gamma-knife treatment have been frequently associated to deafness or decrease balance and frequent falls. Moreover, the present patients have presented far less severe side effects comparing to patients already treated in other studies(187, 188).

Lack of side effects is probably related to the treatment regimen used in this study (5 mg/kg every two weeks), which has been proved to give appreciable results in terms of absence of severe side effects. Higher dosages (i.e. 10-15 mg/kg every two weeks) (207), have been associated with a more frequent occurrence of side effects, as grade III-IV proteinuria, severe infections, hypertension.

Patients affected by meningiomas presented comparable results observed in the schwannomas, with 75% of the eight patients treated for 5 years showing a reduction of the volume of the tumours or an arrest of the growth (60% if the total of 10 patients presenting meningiomas at the start of the protocol is considered). The average tumor growth was 17.21% before the treatment and -4.2% and -1.86% after 1 and 5 years, respectively. In two other studies, efficacy on NF2 related meningiomas has been showed to have contrasting figures: in the study by Nunes et al (197), results were limited in time (average of 15 months) and only 29% showed a tumour mass reduction, while in the study of Alanin on 7 patients, only one presented tumour progression, while the other presented an improvement or a stabilization of their disease (198).

Compared to what obtained for VSs or meningiomas, unsatisfactory results were obtained in patients presenting ependymomas: after 5 years of treatment, 40% presented a tumour arrest or shrinkage, and 60% a tumour progression, and the results in terms of average tumour reduction were not statistically significant after 5 years compared with pre-treatment growth-rate. Also in other case series (200), the effects on these tumours were not impressive. An initial effect of bevacizumab is probably related to its direct action on the cystic (fluid) component of spinal ependymomas (201), but this effect is limited on time because the increase of the solid component of the tumours still progresses even during bevacizumab treatment. In another study, a

more evident action of the drug has been showed in ependymomas presenting high density of VEGF-Receptor 2, even if when VEGF-Receptors 1 were absent, no effect was obtained (200) .

Considering only patients enrolled when younger than 18 years of age (i.e. affected by *Wishart-type* or Congenital NF2) [**table 10**], treatment ensued comparable results: tumour shrinkage was more evident in vestibular schwannomas and meningiomas and not significant in spinal ependymomas. Results on hearing assessment were slightly better (after 5 years there was an average increase of hearing of 3.6% compared to -0.4% of the total patients). The two patients affected by congenital NF2 presented a very appreciable response in term of volume shrinkage and in particular in the instrumental and clinical findings related to their deficits. In the only other study in which the effects on children were evaluated (207) all the children or adolescents showed a massive effect in term of reduction of growth-rate or volume shrinkage, but hearing improvement was appreciable only in one patient out of seven. Even in this study, occurrence of side effects in children was slightly minor comparing to the adults.

The issue on continuation of this treatment after 5 years or more is still matter of debate, as many authors suggest to maintain a lower dosage as long as possible, whereas others, given the minor growth rate of these tumours in the adult-age, suggest a particularly aggressive treatment in the early years and a wait-and-see approach later (150, 167, 213).

Combined therapy could overcome this problem, with a minor maintenance dose of bevacizumab needed (i.e. 2.5 mg/kg), in combination with other drugs (185), or with radiotherapy (203).

In the next future, an early diagnosis, especially for patient with familiarity (present in 45% of cases of NF2 [**table 3**]) could allow to perform a very early treatment, even before the onset of tumours. However, currently there are no reports on bevacizumab used in these early stage of the disease, and it should be considered that the occurrence of side effects like proteinuria, delayed wound repair, increase number of infection, could be related to very severe outcomes in the first decade life.

Moreover, genome-based precision medicine, in oncology, as well as permitting early diagnosis, has widened the field of tumour therapy: many tumours are now studied in their genetic background, and in many non-NF2 patients, a *NF2* gene variant or mutation has been found: anecdotal reports (179) are becoming more and more frequent, and molecular based therapy for these tumours becoming an accepted (standardized) treatment. For this reason, reports on the efficacy of these drugs may be useful for a much larger number of patients than those affected by NF2.

3. Conclusions

This study, as other published in the literature, has highlighted the clinical heterogeneity of the NF2 in younger patients and provided information on the

presentation and progression of the disease in Italian patients. Considering those patients (adults and children), treated by bevacizumab, we found a "stabilizing" effect on VSs and meningiomas, whereas spinal cord ependymomas still need other options (i.e. surgery, radiotherapy) to be satisfactory treated. Efficacy was showed in paediatric patients, especially if treated in the first stages of tumour development, as happened for patients 003, who has showed a dramatic recovery in vision related to the shrinkage of his left optic pathway meningioma.

However, bevacizumab cannot be suggested as a definitive cure for the disease, given the lack of evidences of major responses in these patients: only a minority of them has showed a considerable tumour shrinkage and a considerable clinical improvement of their hearing and clinical conditions.

Appendix

Tables and Charts

Feature	If present at or < age 30 years	If present > age 30 years
First-degree relative with NF2 diagnosed by these criteria	**2**	**2**
Unilateral vestibular schwannoma	**2**	**1**[a]
Second vestibular schwannoma	**4**	**3**[a]
One meningioma	**2**	**1**
Second meningioma (no additional points for > than 2 meningiomas)	**2**	**1**
Cutaneous schwannomas (one or more)	**2**	**1**
Cranial nerve tumor (excluding vestibular schwannoma) (one or more)	**2**	**1**
Mononeuropathy	**2**	**1**
Cataratc (one or more)	**2**	**0**

The patient is given points as shown in the table.

[a] Points are not given for unilateral or second vestibular schwannoma if age at diagnosis is more than 70 yr.

- A diagnosis of **definite NF2** is established if the total number of points is 6 or more.
- *NF2* mutation testing *is indicated* if the total number of points is 4 or 5.

  A diagnosis of definite NF2 is established if a constitutional pathogenic *NF2* mutation is found on mutation testing.

  If no constitutional pathogenic *NF2* mutation is found on mutation testing:

  - A diagnosis of **mosaic NF2** is established if mosaicism for a pathogenic *NF2* mutation is found in the blood or no detectable pathogenic *NF2* mutation is found in the blood but the same pathogenic *NF2* mutation is found in two separate NF2-associated tumors.
  - Otherwise, a temporary diagnosis of **possible NF2** is made, pending further clarification. Clarification may occur if the patient is established to have a different condition (e.g., *schwannomatosis* or *multiple meningiomas*) by standard diagnostic criteria or if evolution of the patient's disease over time permits establishing a diagnosis of definite NF2 or mosaic NF2 according to the criteria given above.

Table 1. The Baser criteria for diagnosis of NF2 [2011]

Author	Year	Patients	Volumetric MRI Response	Hearing response	Side effects
Plotkin et al.	2012	31	**55% Reduction** **32% Stable**	**90% Improvement**	50% methrorrhage 35% proteinuria 26% epistaxis 23% fatigue
Mautner et al.	2010	2	**100% Reduction**	**50% Improvement**	Fatigue, epistaxis, hypertension
Eminowicz et al.	2012	2	**100% Reduction**	**100% Improvement**	NR
Riina et al.	2012	3	**33% Reduction** **66% Stable**	**NR**	None
Subbiah et al.	2012	4	**25% Reduction** **75% Stable**	**25% Improvement** **75% Stable**	50% proteinuria and hyperlipidemia
Hawasli et al.	2013	5	**20% Reduction** **80% Stable**	**60% Improvement** **40% Stable**	40% hypertension ; 20% epistaxis; 20% weight-loss; 20% thoracic or abdominal pain
Alanin et al.	2014	12	**50% Stable or reduction**	**25% Improvement**	92% fatigue; 71% oligomenorrea; 67% proteinuria; 33% hypertension; 17% epistaxis. **One patient died after a brain hemorrhage**
Farschtschi et al.	2015	3	**100% Stable or reduction**	**100% Improvement**	100% hypertension; 66,6% proteinuria;
Hochart et al.	2015	7	**42% Reduction** **42% Stable**	**14.3% Improvement** **42.8% Stable**	14.3%: severe hypertension; protenuria; osteomyelitis; epistaxis; malaise; bleeding

Table 2. Results of Bevacizumab on VSs in NF2

		Italian Network	*Literature Review*	p-value <0.05
Generalities	Total Patients *(number)*	25	330	
	Male	37.5%	49.5%	
	Sporadic Inheritance	79.2%	59.5%	*
Symptoms at Onset	Age *(average)*	5	8.1	
	Central Nervous System	64.2%	23.5%	*
	Skin	54.2%	4%	*
	Peripheral Nervous System/Neuropathy	37.5%	6.5%	*
	Decreased hearing / hearing loss	16.7%	11.1%	
	Eye symptoms	12.5%	10.2%	
	Abdominal pain / masses	4.2%	-	
	Vestibular Schwannomas	4.2%	8%	
	Other / Not specified	-	41%	*
Follow-up	N. Cafè-au-lait *(average)*	2.12	1.5	
	Skin tumours	87.4%	9.4 %	*
	VSs (bilateral or unilateral)	70.8%	27.27%	*
	Hearing Loss	41.7%	22.42%	*
	Visual Impairment	45.8%	12.42%	*
	Cataract	33.3%	21.21%	
	Retinal Hamartomas	24%	4%	*
	Headache	-	0.91%	*
	Seizures	12.5%	6.97%	
	Cranial nerve tumours	16.7%	23.9%	
	Cranial nerve-related symptoms	45.8%	16.6%	*
	Brain tumours	33.3%	7.58%	*
	Spinal cord tumours	83.3%	40.91%	*
	Meningiomas	58.3%	20.61%	*
	Peripheral Neuropathy	54.2%	9.7%	*
	Abdominal schwannomas	8.3%	0.91%	
Outcomes (>10 years)	Hearing Loss	58.3%	59.1%	
	Visual impairment	50%	40%	
	Peripheral neuropathy	62.5%	45.4%	*
	Early death (<25 years of age)	12%	25%	*

Table 3 – Results on symptoms at onset, during a 10-year follow-up and outcomes of the NF2 patients from the Italian network and from a literature review. A p-value lower than 0.05 is reported where data can be compared

NF2

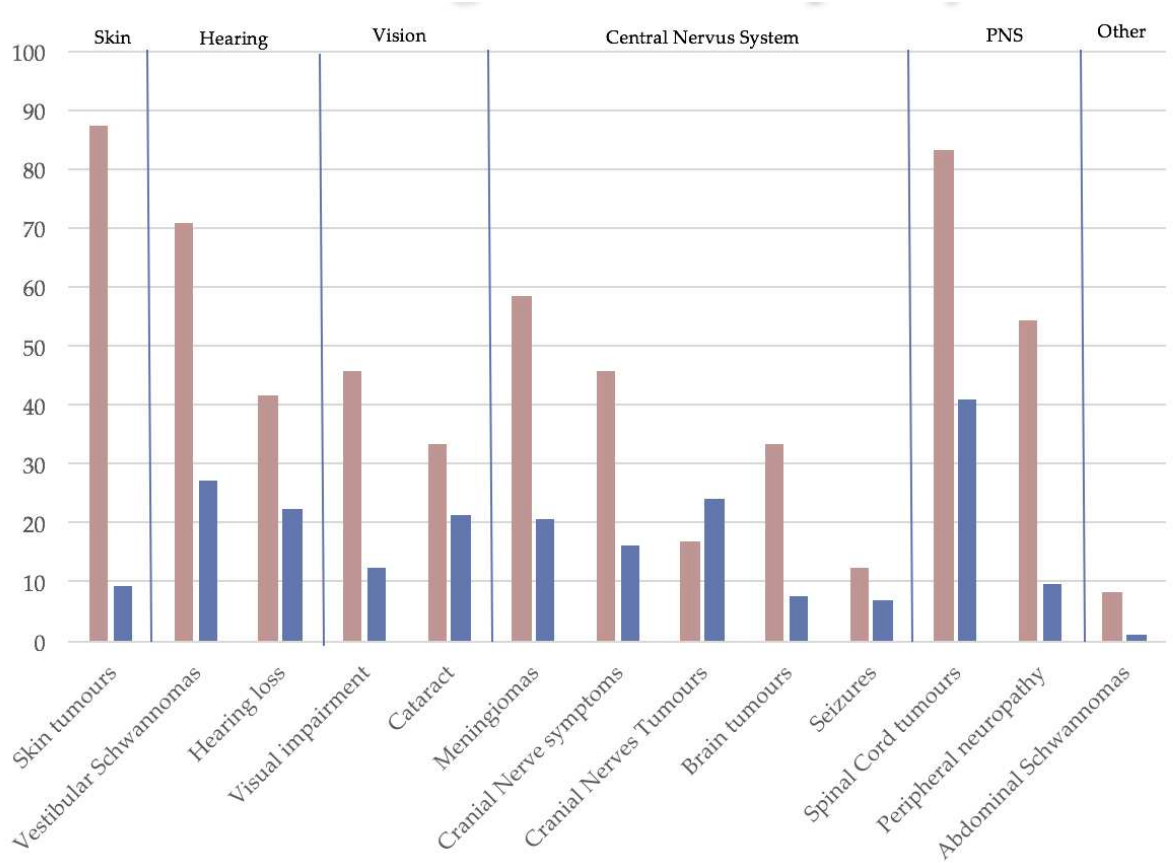

Table 4. A graphical comparison between the present patients (in red) and literature patients (in blue): main findings in a 10-year average follow-up

Patient #ID	Sex	START BEV	END BEV	Age at enrollment	Treatment Duration (years)
#1	F	01/10/12	ongoing	31	5
#2	F	01/10/12	ongoing	22	5
#3	M	01/10/12	ongoing	13	5
#4	M	01/10/12	30/09/13	19	1,5
#5	F	01/10/12	31/03/14	14	1,5
#6	F	01/10/11	01/10/16	16	5
#7	F	29/09/12	19/12/13	28	1,25
#8	M	21/05/13	25/09/14	26	1
#9	F	04/11/13	ongoing	49	4
#10	F	12/12/13	ongoing	20	3,66
#11	M	01/05/13	ongoing	17	4,33
#12	F	01/05/13	ongoing	19	4,33
#13	F	01/09/13	ongoing	22	4
#14	M	01/09/13	ongoing	31	4
#15	F	01/09/13	ongoing	16	4
#16	F	01/09/13	ongoing	22	4
#17	F	01/02/13	ongoing	25	4,58
#18	F	01/02/13	ongoing	21	4,58
#19	F	01/02/13	ongoing	10	4,5
#20	M	01/02/13	ongoing	11	4,5
#21	M	01/10/13	ongoing	28	4,92
#22	F	01/10/13	Ongoing	21	4
#23	M	01/09/12	ongoing	22	5
#24		01/09/12	ongoing	13	5
#25	F	03/09/12	Ongoing	18	5
Total / Average	-	-	-	*21,36*	*3,99*
Standard Deviation	-	-	-	*8,18*	*1,27*

Table 5 – NF2 Patients submitted to Bevacizuamb protocol

Patient #ID	Sex	Pre-treatment shrinkage	1-year shrinkage	5-year shrinkage
#1	F	-22,00	8,33	8,21
#2	F	-19,00	8,13	24,31
#3	M	-17,00	-19,24	-13,26
#4	M	-13,42	35,17	20,92
#5	F	-18,00	-29,96	*Suspended*
#6	F	-32,00	4,66	-29,24
#7	F	-17,45	-37,14	*Suspended*
#8	M	-18,22	-22,86	*Suspended*
#9	F	-15,21	3,83	4,17
#10	F	-17,23	6,52	10,87
#11	M	-19,23	5,89	7,04
#12	F	-16,50	3,94	3,94
#13	F	-45,00	4,64	8,21
#14	M	-23,54	0,70	2,82
#15	F	-21,22	-6,70	-16,20
#16	F	-28,23	-1,82	-16,42
#17	F	-42,11	-15,05	-32,26
#18	F	-48,11	-24,67	-42,00
#19	F	-15,23	3,70	9,88
#20	M	-12,32	8,43	9,64
#21	M	-17,22	3,97	2,38
#22	F	-14,00	0,00	0,00
#23	M	-18,20	6,80	12,00
#24		-12,23	5,19	17,04
#25	F	-14,50	0,71	0,00
Average (SDS)	-	**-21,49 *(10,02)***	**-1,87 *(15,36)***	**-0,36 *(17,41)***
p-value			***P<0,001***	***P<0,001***

Table 6 – Vestibular Schwannomas shrinkage (percentage)

Patient #ID	Sex	Pre-treatment Hearing improvement	1-year hearing improvement	5-year hearing improvement
#1	F	-2,00	6,78	6,78
#2	F	-15,00	29,51	-13,11
#3	M	-12,00	-6,54	-5,61
#4	M	-10,00	-1,68	2,52
#5	F	-12,00	-14,56	
#6	F	-14,00	2,86	-9,52
#7	F	-16,00	0,00	
#8	M	-12,00	0,00	
#9	F	-18,00	0,00	0,00
#10	F	-17,00	10,94	17,19
#11	M	-14,00	1,12	6,74
#12	F	-18,00	-4,84	-1,61
#13	F	-19,00	4,00	11,00
#14	M	-11,00	-1,67	1,67
#15	F	-18,00	-6,25	-7,81
#16	F	-17,00	-2,65	-10,62
#17	F	-23,00	-3,23	-4,84
#18	F	-17,00	-12,50	-18,27
#19	F	-19,00	-2,04	6,12
#20	M	-20,00	13,16	23,68
#21	M	-12,00	-1,92	-3,85
#22	F	-23,00	-12,50	-12,50
#23	M	-16,00	2,83	10,38
#24		-18,00	2,11	7,37
#25	F	-10,00	7,69	7,69
Average (SDs)	-	**-15,32 (4,59)**	**0,42 (9,04)**	**0,61 (10,53)**
p-value			***P<0,001***	***P<0,001***

Table 7. Hearing improvement (percentage)

Patient #ID	Sex	Pre-treatment shrinkage	1-year shrinkage	5-year shrinkage
#1	F			
#2	F			
#3	M	-19,00	19,05	23,81
#4	M			
#5	F			
#6	F			
#7	F	-14,2	2,5	*suspended*
#8	M	-17,2	0	*suspended*
#9	F			
#10	F			
#11	M			
#12	F	-21,40	-0,72	-15,94
#13	F			
#14	M	-17,23	4,88	-2,44
#15	F	-18,2	-5	-12,5
#16	F			
#17	F			
#18	F			
#19	F	-16,55	4,17	7,50
#20	M	-16,22	7,46	8,96
#21	M	-13,20	3,33	3,33
#22	F			
#23	M			
#24		-18,90	6,00	2,20
#25	F			
Average (SDs)	-	-17,21 (2,38)	4,17 (6,37)	1,86 (12,58)
p-value			**P<0,001**	**P<0,001**

Table 8. Meningiomas shrinkage before and after treatment (percentage)

Patient #ID	Sex	Pre-treatment shrinkage	1-year shrinkage	5-year shrinkage
#1	F			
#2	F			
#3	M			
#4	M			
#5	F			
#6	F			
#7	F	-23,00	6,25	
#8	M	-14,50	2,94	
#9	F			
#10	F			
#11	M			
#12	F			
#13	F	-17,20	6,76	-5,41
#14	M			
#15	F			
#16	F			
#17	F	-12,50	3,13	-6,25
#18	F			
#19	F			
#20	M	-27,50	-16,67	-26,67
#21	M			
#22	F	-14,20	0,42	0,00
#23	M			
#24				
#25	F	-12,10	0,00	1,92
Average (SDs)	-	-17,29 (5,83)	0,40 (7,96)	-7,28 (11,38)
p-value			**P<0.001**	**NS**

Table 9. Spinal ependymomas shrinkage, before and after treatment (percentage)

Patient Generalities						Vestibular Schwannomas			Meningiomas			Spinal cord Ependymomas			Hearing Assesment		
Patient #ID	**Sex**	**START BEV**	**END BEV**	**Age enrollment**	**Treatment Duration (years)**	**Pre-Treatment Shrinkage**	**% Shrinkage 1 year**	**% Shrinkage Follow-up**	**Pre-Treatment shrinkage**	**% Shrinkage 1 year**	**% Shrinkage Follow-up**	**Pre-Treatment shrinkage**	**% Shrinkage 1 year**	**% Shrinkage Follow-up**	**Pre-treatment improvement**	**Average improvement 1 year**	**Average improvement Follow-up**
#3	M	**01/10/12**	ongoing	13	5	-17,00	-19,24	-13,26	-19,00	19,05	23,81				-12,00	-6,54	-5,61
#5	F	**01/10/12**	**31/03/14**	14	1,5	-18,00	-29,96								-12,00	-14,56	
#6	F	**01/10/11**	**01/10/16**	16	5	-32,00	4,66	-29,24							-14,00	2,86	-9,52
#11	M	**01/05/13**	ongoing	17	4,33	-19,23	5,89	7,04							-14,00	1,12	6,74
#15	F	**01/09/13**	ongoing	16	4	-21,22	-6,70	-16,20	-18,2	-5	-12,5				-18,00	-6,25	-7,81
#19	F	**01/02/13**	ongoing	10	4,5	-15,23	3,70	9,88	-16,55	4,17	7,50				-19,00	-2,04	6,12
#20	M	**01/02/13**	ongoing	11	4,5	-12,32	8,43	9,64	-16,22	7,46	8,96	-27,50	-16,67	-26,67	-20,00	13,16	23,68
#24	M	01/09/12	ongoing	13	5	-12,23	5,19	17,04	-18,90	6,00	2,20				-18,00	2,11	7,37
#25	F	**03/09/12**	ongoing	18	5	-14,50	0,71	0,00				-12,10	0,00	1,92	-10,00	7,69	7,69
Average (SDs)	**-**	-	-	**14.22 (2.73)**	**4.31 (1.12)**	-17,97 (6.07)	-3,04 (13.22)	-1,89 (16.01)	-17,77 (1.31)	**6,34 (8.60)**	**5,99 (13.08)**	-19,80 (10.89)	-8,33 (11.79)	-12,37 (20.22)	-15,22 (3.60)	-0,27 (8.24)	**3,58 (10.94)**
P value							**P<0.001**	**P<0.001**		**P<0.001**	**P<0.001**		**NS**	**NS**		**P<0.001**	**P<0.001**

Table 10 – Patients affected by NF2 with onset in childhood or adolescence treated by bevacizumab.

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