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Subtype distribution, clinical presentation, and molecular spectrum of neurofibromatosis type 1-associated breast cancer

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ABSTRACT

Aim: To investigate clinical and molecular features of neurofibromatosis type 1 (NF1)-associated breast cancer (BC) in a large multicenter cohort.
Methods: Clinical and histopathological data from 86 NF1 patients with BC (69 with molecular data) were collected, and 111 published cases were reviewed. *NF1* variants were assessed in silico, and their distribution across neurofibromin domains was compared with the general NF1 population.

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Genotype-phenotype correlation
Dominant negative effect
Surveillance

Results: NF1 patients developed BC earlier than the general population (mean 49 years), with missense variant heterozygotes showing the earliest onset (43.9 vs. 49.5 years for truncating variants, $p = 0.014$). Tumors were frequently high-grade (49 %), HER2-enriched (31 %) or luminal B subtypes (31 %), with reduced luminal A (28 %) frequency. NF1+BC patients had more subcutaneous ($p = 0.006$) and plexiform neurofibromas ($p < 0.00001$). Compared with the general NF1 population, they lacked large deletions (0 % vs. 3 %, $p = 0.0148$), showed enrichment for N-HEAT missense variants (70 % vs. 42 %; $p = 0.0078$), and carried recurrent variants significantly enriched in NF1+BC. Structural modeling predicted deleterious effects for >70 % of variants, with proline/arginine substitutions accounting for 83 % of missense variants (vs. 44 % in the general NF1 population, $p = 0.0012$).
Conclusions: NF1-associated BC is characterized by earlier onset, aggressive tumor features, and distinct mutational patterns.

1. Introduction

Neurofibromatosis type 1 (NF1; MIM 162200) is a relatively common disorder with a birth incidence of 1 in 1900–3000 and prevalence of around 1 in 2000–4000 [1,2]. It is caused by heterozygous pathogenic variants in the *NF1* gene (MIM 613113; HGNC:7765), encoding neurofibromin, a 2818-amino-acid protein involved in multiple signaling pathways, including Ras-MAPK, PI3K-AKT-mTOR, and cAMP-PKA [3]. Most pathogenic variants lead to loss of function (LoF) via premature truncation or gene deletion (*NF1* Leiden Open Variation Database (LOVD): https://databases.lovd.nl/shared/variants/NF1). Due to its role in Ras signaling, NF1 is classified as a RASopathy, a group of cancer-prone developmental disorder [4].

Neurofibromin is ubiquitously expressed, especially in the central nervous system, and exists in two main isoforms. The full three-dimensional structure of neurofibromin reveals four major regions: the N-HEAT domain (AA 1–1197)–containing a cysteine- and serine-rich domain (CSRD; AA 543–909) and a tubulin-binding domain (TBD; AA 1095–1197)–the GAP-related domain (GRD; 1198–1535), the SEC-PH region (1536–1817)—comprising a Sec14 homologous (SEC; 1550–1698) and a pleckstrin homology (PH; 1715–1816) domain—and the C-HEAT domain (1818–2818), which includes the C-terminal domain (CTD; 2260–2818). Neurofibromin forms a high-affinity homodimer via a central dimerization module (CDM; 1830–2170), adopts a head-to-tail arrangement of HEAT domains, and transitions between a closed (auto-inhibited) and open (active) conformation [5].

NF1 shows extreme phenotypic variability influenced by mosaicism, modifier genes, and environmental factors. Core features include café-au-lait macules, neurofibromas, freckling, Lisch nodules and choroidal abnormalities; other manifestations include optic gliomas, skeletal abnormalities, learning disabilities and cancer predisposition. Diagnostic criteria were revised in 2021 [6]. Life expectancy is reduced mainly due to neoplastic complications [7].

Genotype–phenotype correlations are increasingly recognized. Large deletions are typically associated with severe presentations [8], while some recurrent variants (*e.g.*, p.Met992del, p.Arg1038Gly, p.Arg1809Cys and splicing variants leading to in-frame skipping of exon 24) are linked to milder phenotypes or Noonan syndrome-like traits [9–13]. In contrast, variants affecting codons 844–848 in the CSRD or codons within the GRD (*e.g.*, p.Arg1276, p.Lys1423) are associated with increased tumor burden and systemic complications [14,15].

NF1 is associated with a markedly elevated cancer risk, including central nervous system tumors, malignant peripheral nerve sheath tumors (MPNSTs), pheochromocytoma, gastrointestinal stromal tumors (GISTs), and early-onset breast cancer [16]. Women with NF1 face a three-to five-fold increased risk of BC, especially before age 50, and tend to have worse outcomes [17]. Somatic *NF1* mutations are also frequent in sporadic BC, supporting a tumor-suppressive role [18].

Despite this, the molecular basis of NF1-associated BC remains poorly understood. Emerging evidence indicates that specific *NF1* variants may modulate BC risk [14,19], and recent findings suggest that certain amino acid substitutions, such as p.Leu847Pro, can destabilize neurofibromin and exert dominant-negative effects through neurofibromin dimerization [20].

In this study, we analyzed the clinical, pathological, and molecular features of BC in a large multicenter cohort of women with NF1. We compared patient characteristics with published cohorts, mapped *NF1* variants to protein domains, and assessed their predicted effects on neurofibromin structure, stability, and dimerization.

2. Materials and methods

2.1. Participants

Medical records of patients with molecularly confirmed NF1 from various referral centers were retrospectively reviewed to identify BC cases. A total of 86 individuals from 85 families were identified, of whom 69 had available molecular data (see Supplementary Table S1).

Data collected included sex, familial history of NF1 and BC, NF1-related features, occurrence of other tumors, and BC characteristics (age at diagnosis, cancer stage, histology, tumor grade, molecular subtype, bilateral occurrence, and relapse, when available). Histopathological data were collected retrospectively, though timing relative to diagnosis or disease progression were unavailable. Reference values for clinical and histopathological BC features were drawn from the literature [21,22].

2.2. Molecular analysis

NF1 molecular analysis was conducted at reference centers for the molecular diagnosis of NF1 (Supplementary Table S1). *NF1* variants were reported according to HGVS nomenclature recommendations (https://hgvs-nomenclature.org/stable/recommendations/general/), based on the *NF1* reference sequence, NM_000267.3. Variant classification followed the guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) [23].

2.3. Mutation spectrum in individuals with NF1-associated BC

To define the *NF1* mutation spectrum in BC, we integrated the collected data with findings from a literature review (Pubmed, up to June 2023) using MeSH (Medical Subject Headings) terms “neurofibromatosis type 1” and “breast cancer.” Case reports and cohort with *NF1* molecular data were included, yielding eight studies [19,24–30]. These were combined into a cumulative NF1+BC cohort for comparative analysis. See Supplementary Fig. S1 for the review process.

The *NF1* LOVD database (accessed September 21, 2023; 5444 entries, 2233 unique pathogenic/likely pathogenic variants) was used as reference. Variants were classified by predicted functional impact (*e.g.*, nonsense [NS], frameshift [FS], affecting splice site [SS], missense [MS], small deletion/duplications of up to 50 codons; in-frame deletions/duplications; indels [ID], large deletions [LD], and others [OT]) following LOVD and [19]. Special attention was given to variant reclassification

based on functional effect (*e.g.*, MS vs. proven SS; [31]. Variants were mapped to neurofibromin functional/structural domains [5].

2.4. Structural in silico, and statistical analysis methods

Details of *in silico* structural analyses, and statistical methods are available in the Supplementary Methods.

3. Results

3.1. Clinical analyses

We analyzed 86 individuals with NF1 and BC from multiple referral centers. NF1 family history was available for 82: 29 (35 %) had a family history, including one pair of affected sisters, while 53 (65 %) were sporadic. Among 73 with available oncological history, 23 (30.5 %) had a family history of BC; in 14 (59 %) of these, BC occurred in individuals affected by NF1. Age at BC diagnosis was available for 80 individuals (mean 49 years, range 28–75), with 49 (62 %) diagnosed at ≤50 years. Six participants (7.7 %) experienced a second BC event: two had homolateral recurrences (5 and 14 years after the first diagnosis), one had synchronous homolateral BC, and three developed contralateral BC (two synchronous, one metachronous). Detailed demographic, molecular, anatomopathological, and clinical data are provided in Supplementary Table S2.

3.2. Anatomopathological analyses

Histology was available for 63 tumors from 59 patients (Table 1, Supplementary Table S2). Invasive ductal carcinoma was predominant (53/63, 84.1 %), consistent with the distribution observed in the general population [32]. Among 35 tumors with grading available, 49 % (17/35) were G3. Compared to the general BC population [21], NF1+BC showed a significantly different subtype distribution ($p < 0.00001$) with lower luminal A (28 % (11/39) vs. 60 % (1787/2984); $p = 0.000062$) and enrichment of HER2+ (31 % [12/39] vs. 7 % [221/2984]; $p < 0.00001$) and luminal B (HER2-) subtypes (18 % (7/39) vs. 8 % (227/2984) (Table 2).

NF1+BC was more aggressive, frequently diagnosed before age 50 (57 % [12/21] of luminal B/HER2+ cases) (Supplementary Table S2) [21]. Compared to other monogenic BC syndromes, NF1+BC had fewer HR + HER2-tumors (10 %), an elevated prevalence of HR- HER2+ (32 %), and few triple negative cases (10 %). HER2+ prevalence was similar to TP53-BC (47 % [NF1+BC] vs. 47 % [TP53-BC]), but NF1+BC had more HR- HER2+ tumors (32 %), while TP53-BC showed more HR + HER2+ tumors (34 %) (Table 3) [22].

Table 1
Breast cancer anatomopathological characteristics at diagnosis in NF1+BC Italian cohort.

Hystotype (n = 63)	N (%)
Invasive ductal carcinoma	53 (84.1)
In situ ductal carcinoma	3 (4.7)
Invasive lobular carcinoma	6 (9.5)
In situ lobular carcinoma	1 (1.5)
Grade (n=35)	**N (%)**
Grade 1	2 (5.7)
Grade 2	16 (45.7)
Grade 3	17 (49.6)
Receptors (n=56)	**N (%)**
Estrogen receptor positive (ER+)	36 (64.3)
Progesterone receptor positive (PR+)	33 (58.9)
Human epidermal growth factor receptor positive (HER2+)[a]	19 (38.8)
Triple-negative	4 (7.1)
Proliferation index Ki-67 (n=39)	**N (%)**
Low Ki-67 (<30 %)	23 (59)
High Ki-67 % (>30 %)	16 (41)

[a] Out of 49 cases tested for HER2.

Table 2
Comparison between the prevalence of St. Gallen surrogate intrinsic BC subtype prevalence in the NF1+BC population compared to the general population. Significant *p*-values (<0.05) are highlighted in bold.

Subtype[a]	NF1+BC population[b]	General BC population[c]	*p*-value
Luminal A	11/39 (28 %)	1787/2984 (60 %)	**0.000062**
Luminal B (HER2-)	7/39 (18 %)	227/2984 (8 %)	**0.016348**
Luminal B (HER2+)	5/39 (13 %)	349/2984 (12 %)	0.828173
HER2 subtype	12/39 (31 %)	221/2984 (7 %)	**<0.00001**
Triple negative	4/39 (10 %)	403/2984 (14 %)	0.554803
Cumulative p-value			**<0.00001**

[a] Ki-67 expression was classified as low for values below 30 % and high for values equal to or above 30 %.
[b] Current study.
[c] [21].

3.3. Clinical characteristics of the NF1+BC cohorts

The clinical phenotype was broadly consistent with the general NF1 population, but subcutaneous neurofibromas ($p = 0.006329$) and pNFs ($p < 0.00001$) were more frequently detected (Table 4). Additional tumors were reported in 26.7 % (23/86) with MPNSTs and GISTs being the most common (5 each, 26 %) (Table 5).

To explore possible associations between tumor burden and BC risk, we analyzed an unselected subcohort of 303 individuals with NF1 (177 women) from Sapienza University (Rome, Italy). Among women with pNFs, 17 of 86 (19.8 %) developed BC, compared to 10 of 91 (11.0 %) without pNFs. Moreover, 3 of 4 women with MPNSTs (75 %) developed BC, versus 24 of 173 (13.9 %) without MPNSTs ($p = 0.0186$), supporting a potential link between tumor burden and increased BC risk.

3.4. NF1 molecular spectrum in NF1-associated BC

Among 71 NF1+BC individuals, causative *NF1* variants were identified in 69 (97 %): 65 classified as pathogenic/likely pathogenic (P/LP), and four as VUS per ACMG/AMP guidelines [23]. A total of 63 unique variants were detected, including 25 novel and 38 reported in LOVD. Most were truncating variants; two multiexonic deletions were found via MLPA, with no whole-gene deletions detected (see Supplementary Table S3 for details).

Combined with literature data (n = 111) [19,24-30], we analyzed 180 NF1+BC cases (Supplementary Table S4). Pathogenic variants were distributed across the whole gene (Fig. 1). Eighteen individuals carried splicing-affecting variants within the *NF1* coding region and were therefore categorized as SS (Supplementary Table S4). Compared to the general NF1 cohort (LOVD, n = 5444) NF1+BC cases had fewer large deletions (0/180 vs. 142/5444; $p = 0.0148$). Missense variants within the N-terminal HEAT domain were significantly enriched in NF1+BC cases (16/23 [70 %] vs. 336/805 [42 %]; $p = 0.007775$), as were variants in the CSRD (12/23 [52 %] vs. 192/805 [24 %]; $p = 0.029311$). Several individual variants were significantly enriched (Supplementary Table S5).

3.5. NF1 variants and breast cancer age at onset

In line with previous reports [19], missense variants were associated with earlier BC onset. Mean age was lower for individuals with missense vs. truncating variants (43.9 vs. 49.5 years; $p = 0.014$; Cohen's $d = 0.587$) (Fig. 2 and Table 6).

Table 3
Comparisons between the distribution of NF1+BC surrogate intrinsic subtypes in NF1-related breast cancers in the current study and those of other reported monogenic BC predisposition syndromes as reported in Ref. [22].[a]

Gene	HR + HER2- Low Grade (n/N (%))[b]	HR + HER2- High Grade (n/N (%))	HR + HER2+ (n/N (%))	HR- HER2+ (n/N (%))	Triple Negative (n/N (%))	*p* - value
NF1[c]	14/41 (34 %)	4/41 (10 %)	6/41 (15 %)	13/41 (32 %)	4/41 (10 %)	
TP53 PTV/MSV[d]	1217/4200 (29 %)	742/4200 (18 %)	1410/4200 (34 %)	540/4200 (13 %)	291/4200 (7 %)	0.001405
PALB2	8739/23300 (38 %)	5909/23300 (25 %)	3130/23300 (13 %)	1521/23300 (7 %)	4001/23300 (17 %)	<0.00001
Non-carriers	2,171,576/3,772,800 (58 %)	522,590/3,772,800 (14 %)	459,426/3,772,800 (12 %)	221,843/3,772,800 (6 %)	397,365/3,772,800 (11 %)	<0.00001
BRCA1 total	7598/48700 (16 %)	7694/48700 (16 %)	1455/48700 (3 %)	3316/48700 (7 %)	28,637/48700 (58 %)	<0.00001
BRCA2 total	28,373/66,200 (43 %)	16,973/66,200 (26 %)	6998/66,200 (11 %)	2307/66,200 (4 %)	11,549/66,200 (17 %)	<0.00001
ATM	12,717/25,000 (51 %)	7886/25,000 (32 %)	2355/25,000 (9 %)	641/25,000 (3 %)	1401/25,000 (6 %)	<0.00001
CHEK2	36,230/61,000 (59 %)	9720/61,000 (16 %)	8919/61,000 (15 %)	2944/61,000 (5 %)	3187/61,000 (5 %)	<0.00001
RAD51C	1603/4200 (38 %)	716/4200 (17 %)	47/4200 (1 %)	270/4200 (6 %)	1564/4200 (37 %)	<0.00001
RAD51D	1500/4500 (33 %)	1458/4500 (32 %)	141/4500 (3 %)	40/4500 (1 %)	1361/4500 (30 %)	<0.00001
BARD1	2269/5200 (44 %)	344/5200 (7 %)	88/5200 (2 %)	394/5200 (8 %)	2105/5200 (40 %)	<0.00001

HR, hormone receptor; HER2, Human Epidermal Growth Factor Receptor 2; n, number of positive tumors; N, total number of examined tumors.
[a] Surrogate intrinsic subtypes as reported in Ref. [22] are classified as follows: i. HR + HER2- Low Grade: ER+ and/or PR+, HER2-, G1 or G2; ii. HR + HER2- High Grade: ER+ and/or PR+, G3; iii. HR + HER2+: ER/PR+ and HER2+; iv. HR- HER2+: ER/PR- and HER2+; v. Triple Negative: ER- PR- HER2-.
[b] Low grade category includes both histological grade 1 and intermediate grade 2 tumors.
[c] Current study.
[d] TP53 PTV/MSV, TP53 protein truncating variants/missense variants.

Table 4
Comparison of clinical features of individuals with NF1+BC (present cohort) with large-scale previously reported cohorts of individuals with "classic" NF1.

NF1 Clinical Feature	NF1+BC cohort Present/Total (%)	Classic NF1[a] Present/Total (%)	p-value
Café-au-lait macule	75/79 (95 %)	1537/1728 (89 %)	0.09335
Skinfold freckling	67/77 (87 %)	1403/1667 (84 %)	0.501682
Cutaneous neurofibromas	70/76 (92 %)	1852/2051 (91 %)	0.599985
Subcutaneous neurofibromas[b]	57/77 (74 %)	297/515 (58 %)	**0.006329**
Plexiform neurofibromas[c]	38/72 (53 %)	120/648 (19 %)	**<0.00001**
Lisch nodules	37/53 (70 %)	729/1237 (59 %)	0.114318
Optic Glioma	6/73 (8 %)	70/519 (13 %)	0.207696
Skeletal dysplasias	8/76 (10 %)	144/1980 (15 %)	0.287421
Scoliosis	19/77 (25 %)	51/236 (22 %)	0.57514
Noonan-like features	0/67 (0 %)	57/1683 (3 %)	0.1677

[a] [11].
[b] In individuals ≥19 years old.
[c] In individuals ≥9 years old.

Table 5
Tumor prevalence in individuals with NF1+BC (present cohort).

Tumor type	N. of cases Present/Total	Prevalence
NF1+BC with at least one other tumor	23/86	26.7 %
NF1+BC with more than one other tumor	4/86	4.6 %
Malignant peripheral nerve sheath tumors	5/86	5.8 %
Gastrointestinal stromal tumors and other soft tissue neoplasia	7/86[a]	8.1 %
Neuroendocrine tumors	4/86	4.6 %
CNS tumors	1/86	1.2 %
Optic Glioma	7/86	8.1 %
Other tumors	4/86	4.7 %

CNS, central nervous system; N., number.
[a] Soft tissue neoplasias included five gastrointestinal stromal tumors and two soft tissue neoplasias of another type.

3.6. *In-silico structural analysis*

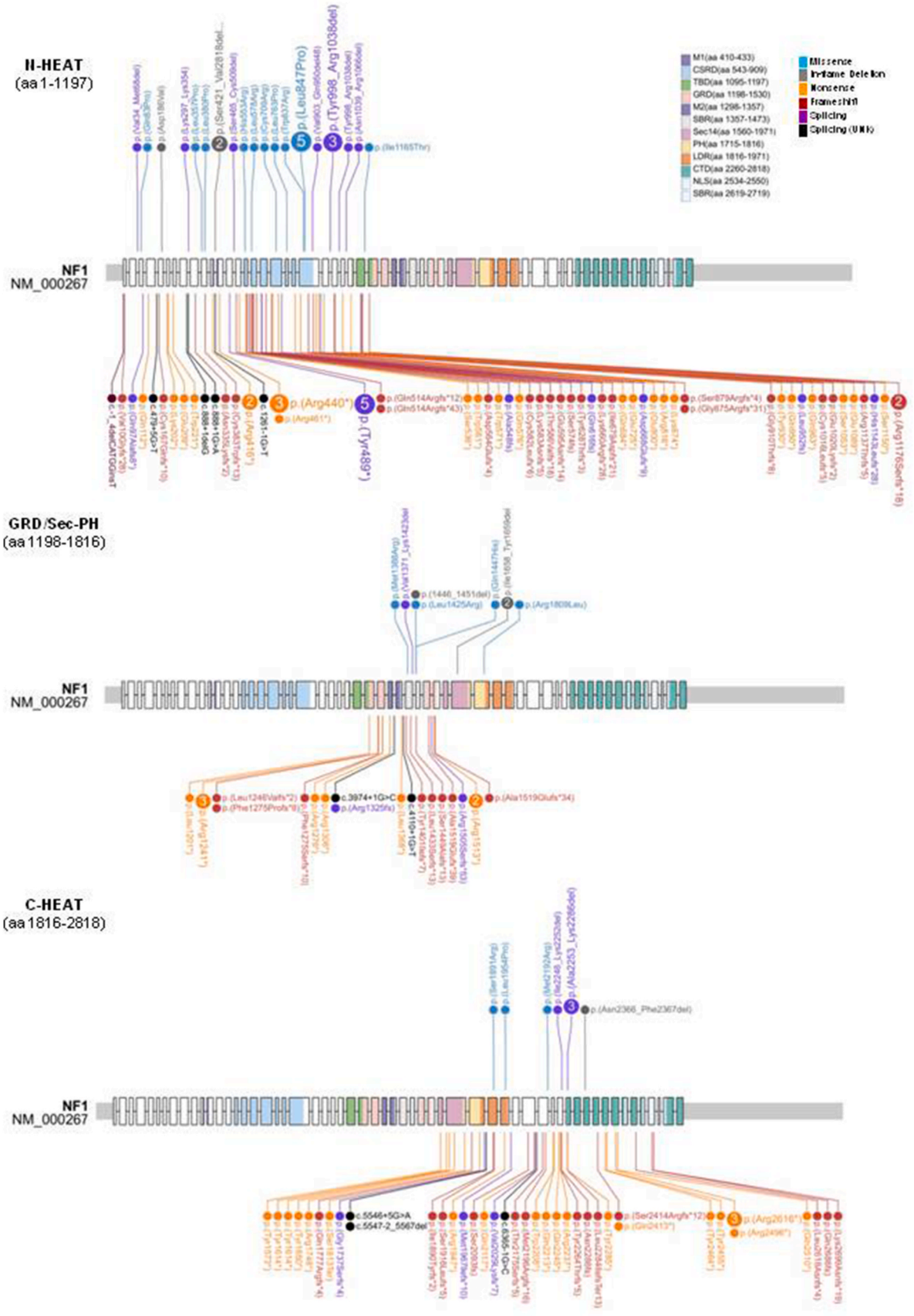
Missense variants affecting NF1 codons 844–848 are associated to severe phenotypes and significant tumor risk [14,19]. Among NF1+BC missense variants, 83 % involved proline (48 %) or arginine (35 %), significantly enriched compared to LOVD (both 22 %; p = 0.0012).

Missense3D predicted 63 % of variants as structurally deleterious, often due to introduction of bulky/charged residues into buried regions. Seven were predicted neutral (Fig. 3A, Supplementary Table S6). FoldX indicated destabilization for 63 % of variants; the most destabilizing included p.Leu847Pro (ΔΔG = 20.53 kcal/mol), p.Leu357Pro (16.55), p.Ser1891Arg (15.27), p.Leu1954Pro (14.53), and p.Trp837Arg (14.35). Six variants, including p.Leu1425Arg (−15.04), were predicted to reduce flexibility (Fig. 3B).

4. Discussion

In this study, we analyzed BC features in a large multicenter NF1 cohort, examined NF1-related phenotypes and complications, and investigated the mutation spectrum alongside the predicted structural impact of *NF1* variants.

Consistent with previous studies, our findings confirmed an increased risk of early-onset BC in women with NF1 (median age 47 and 49 years) [17,33–36] and high rate of contralateral tumors [37]. A



(caption on next page)

Fig. 1. Germline pathogenic *NF1* variants in NF1 and breast cancer cohort by neurofibromin structural domains. This figure illustrates germline pathogenic *NF1* variants identified in individuals with NF1 and breast cancer, stratified by their localization within neurofibromin structural domains. Panels are organized from top to bottom to show the distribution of germline variants along the human *NF1* gene, specifically in the N-HEAT domain (AA 1–1197), GRD-SEC-PH domains (AA 1198–1817), and the C-HEAT domain (AA 1818–2818). Each numbered circle corresponds to the total number of individuals carrying a specific variant. The figure was generated using ProteinPaint (https://proteinpaint.stjude.org/). Variants are represented as follows: missense variants and in-frame indels are displayed above the gene, while variants likely leading to a truncated protein (*e.g.*, nonsense, frameshift, and intronic variants) are shown below. Font colors indicate variant types: missense (blue), indels (gray), frameshift (red), intronic (black), and nonsense (orange).

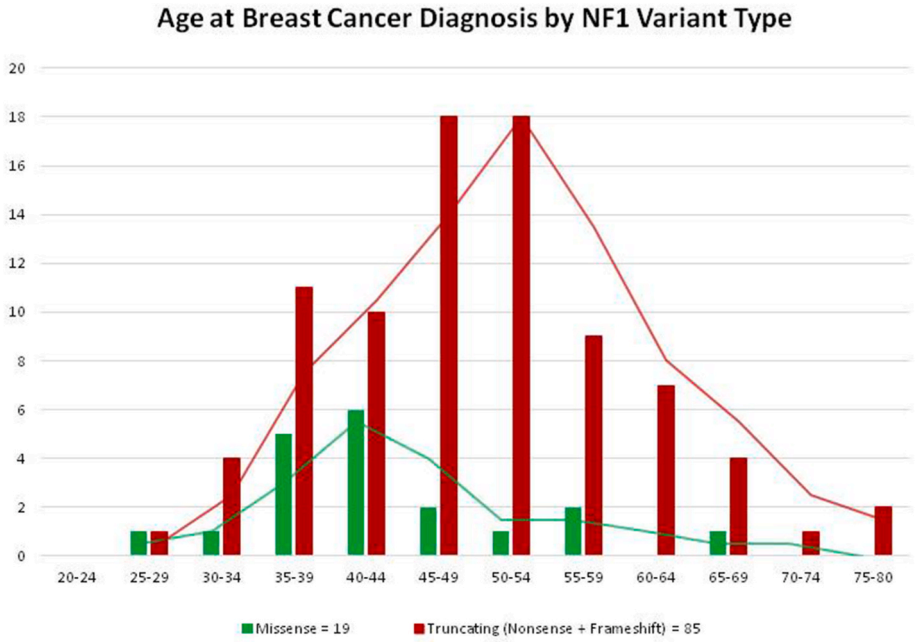


Fig. 2. Age distribution of female breast cancer cases in individuals with *NF1* missense variants versus those with truncating variants. Age is grouped in 5-year intervals. The number of individuals with missense variants is shown in green, while the number of individuals with truncating *NF1* variants is shown in dark red for each age interval.

Table 6
Comparison of age at breast cancer diagnosis in individuals with NF1 missense versus truncating variants.

Variant Types	Missense variants	Truncating variants[a]	Significance[b]
N. of cases	19	85	
Mean	43.8947	49.529	$p = 0.014495$ Cohen's $d = 0.586705$
Median	42	49	
Mode	39	51	
Min.	29	29	
Max.	66	76	
Q_1	39	42.5	
Q_2	42	49	
Q_3	49	56.5	
Range (IQR)	10	14	
Outliers[c]	66	none	

IQR (range), interquartile range, representing the spread of the middle 50 % of age values; Max, maximum age of onset observed; Mean, average age of onset for each variant type; Median, median age of onset; min, minimum age of onset observed; mode, most frequently occurring age(s) of onset; N, number; Q1 (first quartile), the 25th percentile of age of onset; Q2 (second quartile/median), the 50th percentile of age of onset; Q3 (third quartile), the 75th percentile of age of onset.

[a] Truncating variants included nonsense variants and frameshift variants.

[b] Student t-test: statistical test used to compare means between groups.

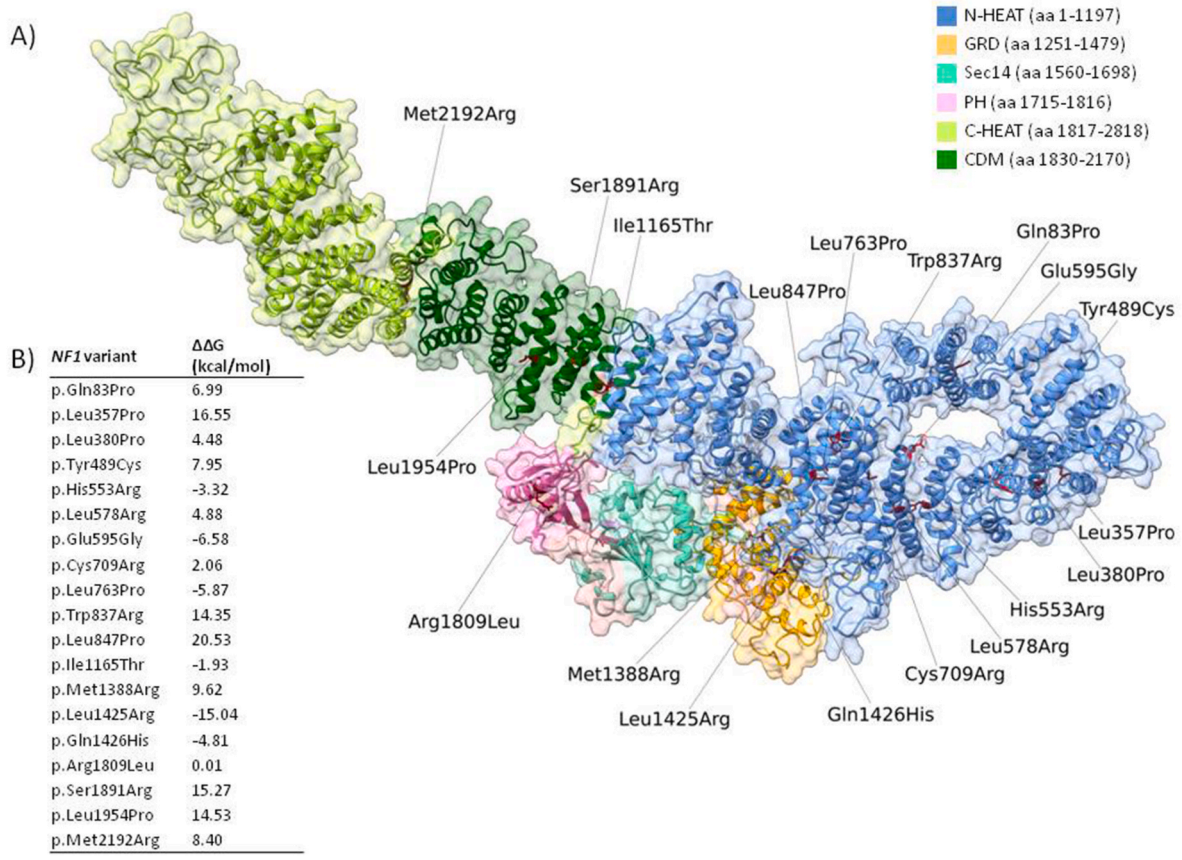
[c] Outliers were defined, for each variant type, as values exceeding the upper fence limit, calculated as Q3 + 1.5 × IQR.

substantial proportion of patients had a history of developing a second mammary tumor. Given that neurofibromin function as a RAS-GAP and modulates estrogen signaling, acting as an estrogen receptor co-repressor [38], hormonal influences may play a role in the age distribution during the fertile period.

Histologically, NF1-associated BC showed unfavorable features, including high grade and elevated proliferation, and frequent *ERBB2* overexpression/amplification, aligning with previous studies on smaller cohorts [39]. Notably, *NF1* depletion has been linked to *ERBB2* overexpression/amplification in sporadic BC with somatic *NF1* variants [40]. This co-occurrence may reflect their proximity on chromosome 17 (*NF1* at 17q11.2 and *ERBB2* at 17q12), predisposing both to joint chromosomal rearrangements [39]. Alternatively, it might be inferred that *NF1* LoF may promote chromosomal instability and *ERBB2* amplification by impairing spindle assembly and chromosome segregation [41,42].

Careful collection of anatomopathological data enabled us to assess the distribution of intrinsic-like BC subtypes within the studied NF1 cohort and compare it with other cancer predisposition syndromes. Notably, NF1-related tumors displayed a distinctive pattern, with a low frequency of HR+/HER2− low-grade subtypes and a relatively high proportion of HR−/HER2+ tumors. This pattern, resembles that of *TP53*-related cancers, which also frequently exhibit *ERBB2* amplification, and differs markedly from the subtype profiles associated with *BRCA1*, *BRCA2*, or *CHEK2* mutations, as well as from those observed in the general BC population (Table 3), suggesting a unique biological behavior of NF1-related BC.

Clinical characterization of individuals affected with NF1 and BC first allowed to document that BC risk is associated with a more severe



NF1 variant	ΔΔG (kcal/mol)
p.Gln83Pro	6.99
p.Leu357Pro	16.55
p.Leu380Pro	4.48
p.Tyr489Cys	7.95
p.His553Arg	-3.32
p.Leu578Arg	4.88
p.Glu595Gly	-6.58
p.Cys709Arg	2.06
p.Leu763Pro	-5.87
p.Trp837Arg	14.35
p.Leu847Pro	20.53
p.Ile1165Thr	-1.93
p.Met1388Arg	9.62
p.Leu1425Arg	-15.04
p.Gln1426His	-4.81
p.Arg1809Leu	0.01
p.Ser1891Arg	15.27
p.Leu1954Pro	14.53
p.Met2192Arg	8.40

Fig. 3. Impact of NF1 Missense Variants on Neurofibromin Stability in NF1+BC Patients. A) Structural localization of *NF1* missense variants identified in NF1 patients with breast cancer on the 3D structure of neurofibromin isoform 1. The neurofibromin domains are color-coded: N-HEAT domain (aa 1–1197) in blue, central dimerization module (CDM, aa 1830–2170) in dark green, pleckstrin homology (PH) domain (aa 1715–1816) in pink, Sec14 domain (aa 1560–1698) in cyan, GAP-related domain (GRD, aa 1251–1479) in yellow, and C-HEAT domain (aa 1817–2818) in light green. **B)** FoldX (http://foldx.embl.de/) analysis results, which predict the impact of variants on protein stability, folding, and dynamics. Variants with ΔΔG values > 0.70 or < -0.70 are in the range to be classified as destabilizing or stabilizing, respectively.

NF1 phenotype, including a higher incidence of subcutaneous neurofibromas and pNFs. Notably, over 26 % of NF1+BC patients developed a second malignancy, which is consistent with previous reports showing rates between 14 % and 29 % [19,26,34,36] (Supplementary Table S7), and slightly exceeding the 17 % reported in a large apparently unselected NF1 cohort [16]. When combining data from all available NF1+BC cohorts (55/210; 26.2%) [19,26,34,36; current cohort], the frequency of second malignancies was significantly higher than in the reference unselected cohort (244/1404; 17.4%; $p < 0.00001$) (Supplementary Table S7) [16]. Although these findings suggest an increased overall tumor susceptibility in NF1 patients with BC, further studies are warranted to confirm this observation. Comparisons may be affected by age-related differences in phenotype penetrance—our NF1+BC cohort had a median age of 49 years (range 28–75), whereas the comparison cohorts for subcutaneous neurofibromas and pNFs included individuals aged ≥19 years and ≥9 years, respectively—and by variability in tumor detection methods. In particular, superficial pNFs are typically identified by clinical examination, while internal tumors are more often detected by MRI; notably, pNFs have been reported in up to 57 % of children with NF1 who underwent whole-body MRI combined with clinical-neurologic examination) [43]. In our cohort, information on whether tumor assessment was performed through MRI or clinical examination was not available from the retrospectively reviewed clinical records.

Consistent with previous reports [19,24-30], no whole-gene deletions were identified in the present NF1+BC cohort. This may reflect reduced life expectancy in deletion carriers or suggest that BC risk might be related to a dominant-negative behavior or specific functional dysregulation of neurofibromin rather than simply LoF [19]. However, recent identification of three NF1+BC subjects among 126 whole-gene deletion carriers [44], indicates that BC risk in this subgroup cannot be ruled out and warrants further investigation.

In addition to the absence of large deletions, Frayling et al. (2019) noted a predominance of nonsense and missense variants in NF1+BC patients and proposed a potential gain-of-function or dominant negative mechanism [19]. This hypothesis is supported by structural studies showing that neurofibromin functions as a high-affinity dimer [5]. While no enrichment of nonsense variants was observed in our cohort, missense variants in the N-terminal HEAT domain were significantly more frequent in NF1+BC patients compared to the general NF1 population, with a similar enrichment observed for variants localized specifically within the smaller CSRD domain. Two recurrent N-HEAT domain variants (c.1466A > G p.Tyr489* and c.2540T > C p.Leu847-Pro), were significantly enriched in NF1+BC patients (2.78 %) versus the general NF1 population, as reported in the LOVD database (0.83 % and 0.72 %, respectively). The splice-altering c.1466A > G variant introduces a premature stop codon (p.Tyr489*) [45] and has been identified in multiple NF1 individuals with BC [19,26]. Functional analyses in CRISPR/Cas9-edited iPSCs revealed leaky splicing, with approximately 85 % aberrant transcripts and ~15 % full-length transcripts encoding the p.Tyr489Cys protein [45]. Consistent findings were obtained using a minigene assay in an independent study, which confirmed the presence of correctly spliced transcripts containing the r.1466a > g substitution and producing the p.Tyr489Cys protein at appreciable

levels [46]. While these data suggest that the c.1466A > G variant may act through dual mechanisms, namely, a splicing defect and the production of residual mutant protein, neurofibromin was undetectable by Western blot, indicating post-transcriptional or translational mechanisms likely contributing to protein loss [45]. iPSCs harboring this variant also exhibited Ras pathway hyperactivation, evidenced by increased levels of GTP-bound RAS, pERK/ERK, and pS6/S6, with no change in AKT signaling [45]. The p.Leu847Pro missense variant, also within the N-HEAT domain, has previously been associated with a more severe NF1 phenotype and increased malignancy risk [14], and shows significant enrichment in NF1+BC patients [19,24,26]. These findings support the occurrence of genotype-phenotype correlations in NF1-associated BC.

Young et al. (2023) functionally characterized *NF1* variants at codons 844–848, including p.Leu847Pro, showing that this substitution leads to reduced protein levels, increased polyubiquitination and interaction with HSP70, which are hallmarks of protein misfolding [20]. Although capable of heterodimerizing with wild-type neurofibromin, the mutant isoform was observed to cause co-degradation of the wild-type protein, consistent with a dominant-negative effect. Cryo-EM analysis revealed that such variants disrupt α-helical packing and expose buried hydrophobic residues, promoting misfolding and degradation of both isoforms [20].

Supporting this model, 63 % of missense variants in NF1+BC individuals were predicted to destabilize neurofibromin structure, particularly those involving residues spotted in the N-HEAT domain (*e.g.*, p.Gln83Pro, p.Leu357Pro, p.Leu380Pro, p.Tyr489Cys, p.Leu578Arg, p.Cys709Arg, p.Trp837Arg, and p.Leu847Pro), possibly extending to those located within the C-HEAT domain (*e.g.*, p.Ser1891Arg, p.Leu1954Pro, and p.Met2192Arg). Destabilization likely results from the insertion of bulky or charged residues into buried protein regions, impairing proper protein folding and stability. Substitutions often involved proline and arginine residues (48 % and 35 %, respectively), which are significantly enriched compared to the general NF1 population (22 % each in LOVD). This suggest a link between destabilizing substitutions and increased BC risk. Other mechanisms may underlie variants in functional domains like the GRD (*e.g.*, p.Leu1425Arg and p.Gln1426His) or pleckstrin homology domain (*e.g.*, p.Arg1809Leu), with effects ranging from structural rigidity to negligible effect (Fig. 3A and B).

Notably, NF1 patients with missense variants developed BC at significantly earlier age than those with truncating variants (43.9 vs. 49.5, $p = 0.014$), supporting the hypothesis that certain missense variants may accelerate tumorigenesis. This may reflect more severe functional disruption compared to mere haploinsufficiency resulting from truncating variants.

The present study may have some limitations, including its retrospective design, which limited information on tumor assessment methods and age-related comparisons, the use of the LOVD database as a reference for the general NF1 population, which may not fully capture its diversity, and the absence of formal correction for multiple comparisons, which could influence the interpretation of certain associations.

In conclusion, our study confirms an increased risk of early onset BC in women with NF1, underscoring the need for earlier and comprehensive cancer surveillance, as recommended in the Genturis Guidelines [47]. Breast MRI is advised starting from the age of 30 (or mammography when MRI is not available), to be performed yearly between 30 and 50 years of age (strength: moderate). After 50 years, breast screening should follow the national guidelines for the general population.

NF1-associated BC displays worse prognostic features, including frequent HER2/Neu overexpression, and may involve neurofibromin dysfunction beyond RAS-GAP activity. Women with NF1 and BC also show a more severe NF1 phenotype, including increased pNF burden and potential MPNST risk. At the molecular level, BC risk varies across *NF1* variants, with missense variants within the N-terminal HEAT domain being more prevalent and predicted to cause greater protein destabilization. Notably, p.Leu847Pro, one of the most recurrent variants in NF1+BC patients, has been functionally shown to act via a dominant-negative mechanism, supporting a variant-specific contribution to BC susceptibility in NF1.

CRediT authorship contribution statement

Niccolò Di Giosaffatte: Writing – original draft, Visualization, Resources, Investigation, Formal analysis, Conceptualization. **Paola Daniele:** Investigation. **Francesco Petrizzelli:** Visualization, Formal analysis. **Chiara Iacovino:** Resources, Investigation. **Chiara Canciani:** Resources. **Maria Luisa Garau:** Resources. **Claudia Santoro:** Resources. **Valentina Trevisan:** Resources. **Arianna Panfili:** Resources. **Stefania Cavone:** Investigation. **Valentina Guida:** Investigation. **Maria Cecilia D'Asdia:** Investigation. **Laura Bernardini:** Investigation. **Silvia Majore:** Resources. **Alessandro Ferraris:** Resources. **Michele Valiante:** Resources. **Francesca Gensini:** Resources. **Francesca Clementina Radio:** Resources. **Giada Tortora:** Resources. **Matteo Cassina:** Resources. **Giuseppina Miele:** Resources. **Manuela Priolo:** Resources, Investigation. **Fabio Sirchia:** Resources. **Ludovica Piccinno:** Investigation. **Elisabetta Flex:** Investigation. **Giuseppe Zampino:** Resources. **Maurizio Genuardi:** Resources, Investigation. **Vincenzo Nigro:** Resources. **Leonardo Salviati:** Resources, Investigation. **Laura Papi:** Resources, Investigation. **Paola Grammatico:** Resources. **Chiara Leoni:** Resources. **Giulio Piluso:** Investigation. **Sandra Giustini:** Resources. **Tommaso Mazza:** Writing – original draft, Visualization, Investigation, Formal analysis. **Meena Upadhyaya:** Writing – review & editing, Resources. **Marco Tartaglia:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Eva Trevisson:** Writing – review & editing, Supervision, Resources, Investigation, Conceptualization. **Alessandro De Luca:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Data availability statement

The datasets generated and/or analyzed during the current study can be obtained from the corresponding author upon request. All variants have been added to the Leiden Open Variation Database (https://databases.lovd.nl/shared/variants/NF1/unique/).

Ethical approval

The study was performed in accordance with the principles set out in the 1984 Declaration of Helsinki and subsequent versions and was approved by the local institutional review boards (no. prot. 182/CE/2024_AOU Foggia 2024.12.17; no. prot. 5994/AO/24_ AOU Padova 2024.06.28). All institutions involved in human participant research received local Institutional Review Board approval. All patients signed an informed consent for the scientific use of clinical and genetic data.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

All authors have reviewed and approved this declaration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2025.104618.

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