

Status and Recommendations for Incorporating Biomarkers for Cutaneous Neurofibromas Into Clinical Research

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Abstract

Objective

To summarize existing biomarker data for cutaneous neurofibroma (cNF) and to inform the incorporation of biomarkers into clinical trial design for cNFs.

Methods

The cNF working group, a subgroup of the Response Evaluation in Neurofibromatosis and Schwannomatosis (REiNS) consortium, was formed to review and inform clinical trial design for cNFs. Between June 2018 and February 2020, the cNF working group performed a review of existing data on genetic biomarkers for cNFs in the setting of neurofibromatosis type 1. We also reviewed criteria for successful biomarker application in the clinic. The group then held a series of meetings to develop a consensus report.

Results

Our systematic literature review of existing data revealed a lack of validated biomarkers for cNFs. In our report, we summarize the existing signaling, genomic, transcriptomic, histopathologic, and proteomic data relevant to cNF. Finally, we make recommendations for incorporating exploratory aims for predictive biomarkers into clinical trials through biobanking samples.

Conclusion

These recommendations are intended to provide both researchers and clinicians with best practices for clinical trial design to aid in the identification of clinically validated biomarkers for cNF.

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Glossary

cNF = cutaneous neurofibroma; mTOR = mammalian target of rapamycin; NF1 = neurofibromatosis type 1; REiNS = Response Evaluation in Neurofibromatosis and Schwannomatosis.

Neurofibromatosis type 1 (NF1) is an autosomal dominant neurogenetic condition that affects ≈ 1 in every 2,000 to 3,000 births^{1,2} and is characterized by pathogenic germline alterations at the *NF1* locus on chromosome 17. The NF1 phenotype is heterogeneous, but almost all affected individuals develop benign skin tumors called cutaneous neurofibromas (cNFs). Other characteristic features include café-au-lait macules, axillary freckling, and plexiform neurofibromas in addition to CNS abnormalities.

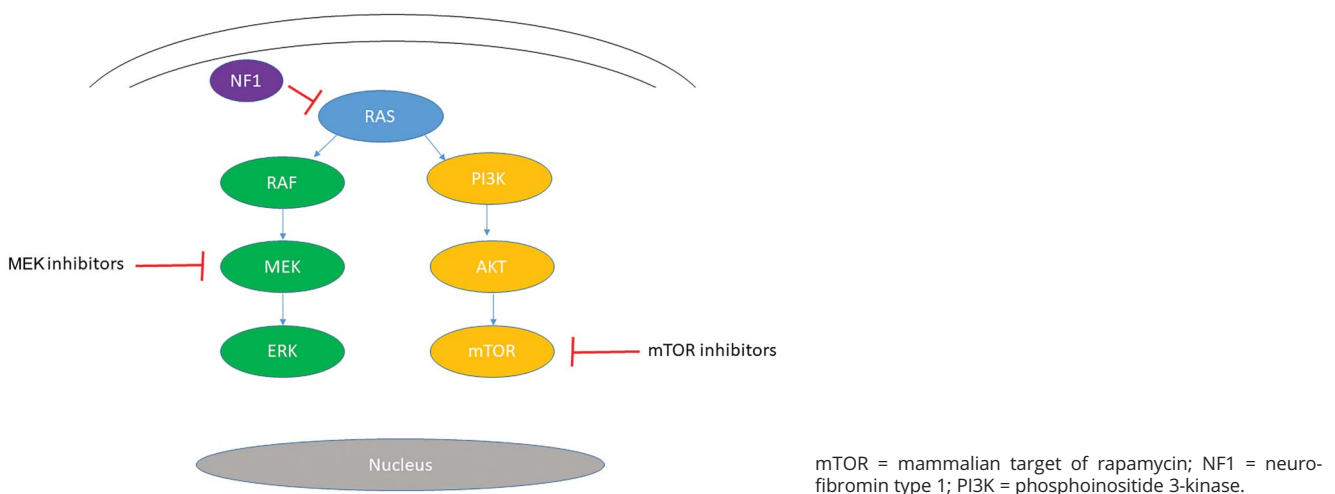
cNFs are mixed cell-type tumors composed of Schwann cells, fibroblasts, macrophages, and mast cells. Although histologically benign, cNFs can cause significant itching, pain, social anxiety, and distress. Affected individuals can have a few to thousands of cNFs and indicate that cNFs are a primary factor in reducing their quality of life.³⁻⁶ cNFs can appear early in life but have their highest rate of development in young adults and increase over time.³ Some studies report rapid growth in puberty and pregnancy.⁷⁻⁹ Treatment of cNFs is currently limited to surgical excision and destruction (e.g., laser or electrodesiccation), which result in scarring and have a high recurrence rate.¹⁰⁻¹⁷ In addition, removing all lesions is not usually feasible due to the abundance of cNFs and the side effects of the available interventions. Thus, there is a growing interest in developing nonsurgical treatments for cNFs.

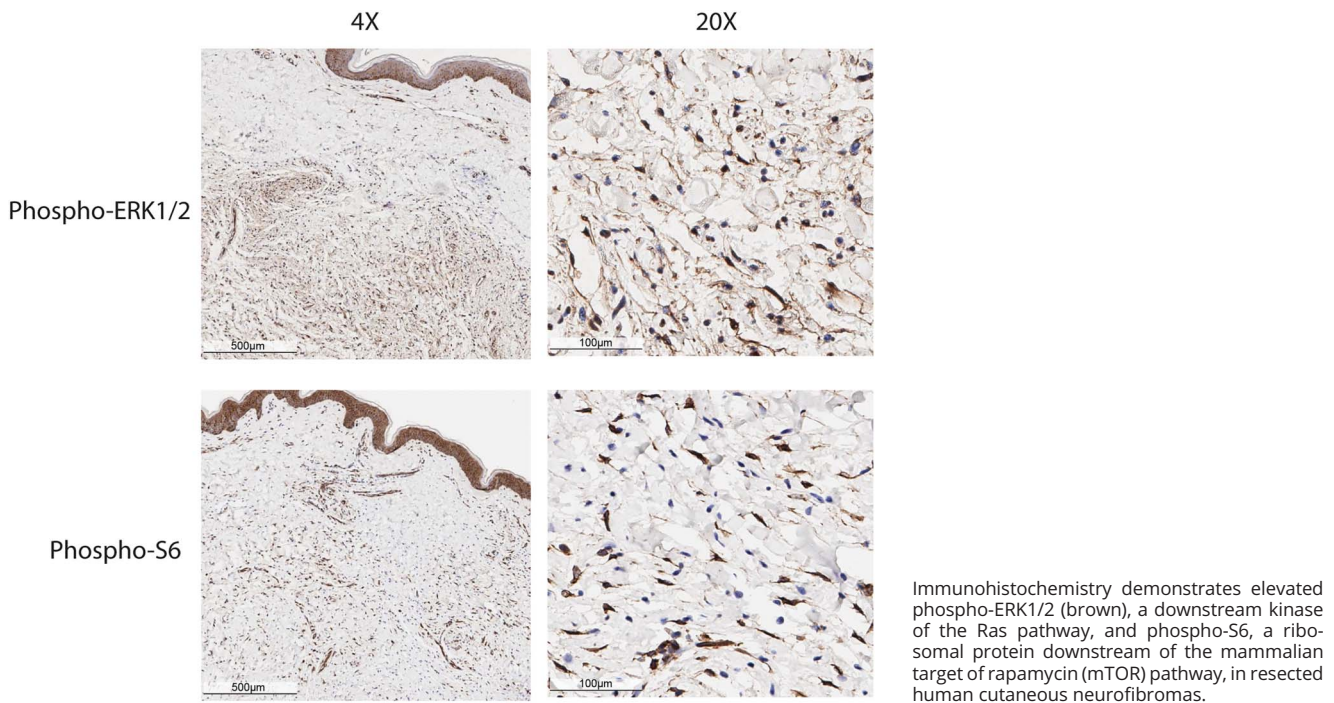
cNFs are driven, at least in part, by uncontrolled activation of the Ras pathway within Schwann cells. Individuals with NF1 have a defect in the *NF1* gene, which encodes neurofibromin, a GTPase activating protein and negative regulator of the Ras

pathway. The primary function of neurofibromin is to bind active *guanosine triphosphate*-Ras and to stimulate Ras GTPase activity to cleave *guanosine triphosphate* into guanosine diphosphate, returning Ras to its inactive guanosine diphosphate-bound state. cNFs harbor biallelic pathogenic variants in *NF1* in Schwann cells, leading to unhindered activation of the Ras signaling pathway (figure 1), which results in cellular overproliferation and tumor formation. Indeed, both the RAF and phosphoinositide 3-kinase pathways are known to be upregulated in cNFs (figure 2), and inhibitors to these pathways are being investigated as therapeutics (e.g., selumetinib inhibits MEK in the RAF pathway and rapamycin inhibits mammalian target of rapamycin [mTOR] in the phosphoinositide 3-kinase pathway) in NF1-associated peripheral nerve sheath tumors.

The Response Evaluation in Neurofibromatosis and Schwannomatosis (REiNS) International Collaboration was created in 2011 to define and develop the most informative, reliable, and meaningful endpoints for clinical trials for NF conditions.¹⁸ The cNF working group of REiNS is focused on understanding the natural history of cNFs, developing clinical trial designs, and identifying meaningful endpoints for trials in cNFs. As part of this effort, a cNF Biomarker subgroup, composed of dermatologists, neurologists, geneticists, oncologists, pathologists, pediatricians, and basic scientists, was formed to investigate the role of biomarkers and to make recommendations for biomarker incorporation into cNF trials. Here, we build on and update the prior REiNS Biomarker working group findings and make recommendations specific to clinical trials for cNFs.

Figure 1 Diagram of Ras and mTOR Pathways in NF1 and Targeted Intervention With MEK and mTOR Inhibitors





Immunohistochemistry demonstrates elevated phospho-ERK1/2 (brown), a downstream kinase of the Ras pathway, and phospho-S6, a ribosomal protein downstream of the mammalian target of rapamycin (mTOR) pathway, in resected human cutaneous neurofibromas.

Methods

Consensus Process

Between June 2018 and February 2020, the Biomarker subgroup of the cNF working group of REiNS performed an extensive literature search and reviewed and summarized data on biomarkers in cNF. The group then had a series of 5 consensus meetings to review the data and to develop consensus recommendations for incorporation of biomarkers into cNF clinical trials. The report was drafted in February 2020 and then circulated to patient representatives, the broader REiNS biomarker working group, and the REiNS director council for comments.

Immunohistochemistry

Deidentified cNF samples were obtained from discarded surgical excision specimens at Stanford University under IRB 24307. Immunohistochemistry for phosphorylated ERK was performed by HistoWiz Inc (Brooklyn, NY). Immunohistochemistry was performed on formalin-fixed and paraffin-embedded 4-µm sections. Primary antibodies used were rabbit phospho-ERK (Cell Signaling, Beverly, MA; 4307S, 1:100) and phospho-S6 (Ser 235236, Cell Signaling; 4858S, 1:100).

Results

Biomarker Definitions and Trial Types

Biomarkers are increasingly being incorporated into clinical trials with promise to aid in the ability to predict and monitor disease and therapeutic response. In 1998, the NIH

Biomarkers Definitions Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” Biomarkers can be obtained from bodily fluids such as plasma, serum, or urine and from tumor tissue. There are currently 3 main categories of biomarkers categorized by their functional capabilities: prognostic, pharmacodynamic, and predictive.

Application of Biomarkers to cNF Clinical Trials

Incorporating Prognostic Biomarkers Into Clinical Trials for cNF

A prognostic (outcome) marker informs the likely outcome of a disease and whether additional therapy would be beneficial. These biomarkers can provide insight into a patient’s disease outcome independently of any specific intervention. Prognostic biomarkers can be incorporated into clinical trials to predict cNF number or growth. For example, up to 5% of individuals with *NF1* harbor microdeletions and are more likely to have high numbers of cutaneous, subcutaneous, and spinal neurofibromas. There are also genotypes associated with no cNFs, including *NF1* deletions at c.2970-2972 delAAT (p.990delM) and mutations at p.R1038G and p.R1809 (see also Plotkin et al.¹⁸ this issue).¹⁹⁻²² While genetic alterations at or near the *NF1* locus explain some of the phenotypic heterogeneity seen across people with *NF1*, the familial aggregation of specific phenotypes suggests the influence of a strong genetic component unlinked to the underlying *NF1* mutation.^{23,24} An understanding of which

individuals are likely to develop high numbers or large cNF could inform enrollment in clinical trials, especially when one is considering prevention studies.

In addition to host information, prognostic biomarkers can be derived from somatic tissues such as cNFs skin biopsies. These are important given the variability of cNF behavior within a given individual. There have been limited studies using sequencing, transcriptomics, and proteomics to discover factors important to cNF pathogenesis (table 1). Initial microarray studies compared cNF and plexiform neurofibroma samples.^{25,26} Recent whole-exome sequencing and pathway analysis of human cNFs revealed somatic mutations in 3 genes—*RASSF1A*, *SFN*, and *DLG4*—that belong to the Hippo pathway.²⁷ A follow-up study analyzed the whole-genome sequencing dataset from 40 cNFs from 11 individual patients with NF1 and found multiple germline mutations in genes involved in the Hippo pathway and high levels of key effectors of the Hippo pathway in cNFs.²⁹ When *Lats1* or *Lats2* (negative regulators of *YAP1*) is perturbed in the *Hoxb7-Nf1* murine model, the cNF burden increases. Such studies help identify targets and pathways for treatment development and potentially determine molecular enrollment criteria for clinical trials and identification of which tumors or patients may benefit from a specific intervention.

Histopathologic data may also serve as prognostic biomarkers for cNF. cNFs express several growth factors and hormone receptors and activate multiple kinase pathways such as the Ras/mitogen-activated protein kinase pathway. Schwann cells in cNF express progesterone receptors³⁰ but not estrogen receptors.³¹ Furthermore, an increase in Schwann cell proliferation rate in vitro was observed after progesterone exposure.³² Increased expression of Ghrelin receptor (thought to

regulate energy homeostasis)³³ has been reported in larger tumors. Midkine is a potent angiogenesis factor, expressed in both cNF Schwann cells and endothelial cells.³⁴ Insulin-like growth factor-1 receptor is also expressed in neoplastic Schwann cells (but more in plexiform than cutaneous tumors).³⁵ Growth hormone receptor is expressed in most cNF associated with NF1.³⁶ Furthermore, subtyping cNFs into categories based on proliferation rate or other factors may help identify new prognostic biomarkers.

Incorporating Pharmacodynamic Biomarkers Into Clinical Trials for cNF

Pharmacodynamic biomarkers are used to assess ability of therapeutics to engage their targets. For example, ERK phosphorylation status can be used to evaluate target engagement of MEK inhibitors and suppression of the RAS/mitogen-activated protein kinase pathway. Signaling and pathway activation studies of plexiform neurofibromas suggest that both RAS-MEK pathway and mTOR are logical therapeutic targets in cNF (figure 1). Pharmacokinetic biomarkers provide information on the disposition of drugs and their metabolites in blood and tissue. Pharmacokinetic assessment has an important role in NF1 clinical trials because the concentrations that cause toxicity with some systemic drugs have been lower than seen in patient populations without NF1.³⁷

Moreover, it may be that lower-than-expected systemic concentrations are needed for target engagement. This was demonstrated in preclinical models in which a lower dose of the MEK inhibitor PD-0325901 (mirdametinib) was as active in both efficacy and pharmacodynamic metrics against plexiform neurofibroma as higher doses.³⁸ Similarly, preclinical pharmacokinetic/pharmacodynamic studies of the multikinase inhibitor cabozantinib revealed variable target engagement

Table 1 Genomics Studies Using cNF Tissue

Material	Technology	Experiment	Key results	Reference
cNFs	Whole-exome sequencing	Whole-exome sequencing of 3 growing and 3 nongrowing cNFs from a single individual to assess the role of acquired somatic mutations in neurofibroma growth behavior	1–11 Mutations were identified in each sample, including 2 deleterious <i>NF1</i> mutations; mutations in the Hippo signaling pathway appeared to be overrepresented	27
cNFs derived from 11 unrelated individuals with NF1	SNP array, whole-genome sequencing, and RNA sequencing	Characterization of ≈40 cutaneous tumors from 11 individuals by SNP array, whole-genome sequencing, and RNA sequencing	Sequencing data available in a public database	28
14 Human plexiform neurofibromas and 10 MPNSTs; 20 dermal neurofibromas	Microarray	Gene expression levels in plexiform neurofibromas and MPNSTs, determined by oligonucleotide microarray and real-time RT-PCR analyses; expression is relative to the expression levels in dermal neurofibromas	<i>TNXB</i> and <i>TNC</i> may be involved in the malignant transformation of plexiform neurofibromas	25
10 Normal Schwann cells, 22 NFSCs, 13 MPNST lines, 26 benign neurofibromas, and 6 MPNST tumors	Microarray	Microarray of 22 NFSCs, 13 MPNSTs, and 26 benign neurofibromas; comparison group consisted of normal Schwann cells	Differential expression of 2,827 transcripts in Schwann cells; identification of <i>SOX9</i> as a biomarker and survival gene	26

Abbreviations: cNF = cutaneous neurofibroma; MPNST = malignant peripheral nerve sheath tumor; NF1 = neurofibromatosis type 1; NFSC = neurofibroma Schwann cell.

dependent on tissue concentration. Some of the preclinical pharmacodynamic markers were validated in the clinical trial for plexiform neurofibroma.^{39,40}

In conclusion, pharmacodynamic markers can be used to assess target engagement of a drug in preclinical and clinical trials and can be analyzed in clinical trials to determine the level of target engagement required for therapeutic effect or on-target side effects.

Incorporating Predictive Biomarkers Into Clinical Trials for cNF

Validated predictive biomarkers have the potential to serve as proof-of concept surrogates in clinical trials. To serve as surrogates for drug activity, predictive markers must meet several criteria. There must be a clear relationship between exposure of the target and the predictive biomarker. If the biomarker can demonstrate that the candidate drug engages the biomarker at a reproducible and measurable level in humans with a reliable functional effect, it can be used in early clinical trials. Alternatively, if a biomarker can demonstrate that the candidate drug results in a biological or clinical change associated with the disease or mechanism of action, it can also be used in early clinical trials. Finally, predictive biomarkers can be used for proof of concept if they show that the candidate drug results in a clinical change on an accepted endpoint in patients with disease.

There is a need to develop immunohistochemical markers of different cellular components of cNF and to define the prevalence of growth factors or activated pathways and involution markers. All these biomarkers would be helpful for the development and assessment of therapies in cNF. Predictive biomarkers may also be used as correlates with clinical outcomes. Examples include using terminal deoxynucleotidyl transferase dUTP nick-end labeling staining to evaluate apoptosis and Ki-67 levels, which are associated with cell proliferation. Early changes may be associated with future clinical outcome predictors.

Suggested Studies to Support Biomarkers in Future NF1 Clinical Trials

On the basis of existing data and emerging technology, the following -omics studies are recommended to support biomarker identification and validation for cNFs. Epigenomic studies should be focused on altered DNA methylation regions in cNF and the study of chromatin accessibility using assay for transposase-accessible chromatin using sequencing. Transcriptomic studies of cNF tissue with comparator non-tumor tissue need to be carried out to delineate molecular pathways altered in cNF. This information can be used to identify patients, and potentially tumors, into molecularly stratified subtypes for targeted therapeutic intervention in future trials. Single-cell RNA sequencing is recommended to better understand the tumor heterogeneity and intercellular interactions. Genomic studies such as exome sequencing can reveal somatic tumor evolution of cNF and provide more data

on genetic heterogeneity. This information can also be used to identify mutations in other driver somatic genes and germline modifiers. More proteomic studies are also needed to examine protein changes within cNF with and without therapeutic intervention, including Schwann cells, mast cells, and other cell types.

In the evaluation of the utility of a potential biomarker, several considerations should be kept in mind. Clinical relevance is key because the biomarker should be related to the disease prognosis, mechanism of action of the drug in humans, or clinical endpoint. The ease of detecting the biomarker or change in biomarker in the target population is also critical, as is the reliability in measuring the biomarker with accuracy, precision, and reproducibility. Furthermore, practicality and simplicity play important roles in multicenter clinical trials. Simple and minimally invasive are optimal conditions for translating a biomarker from laboratory bench to bedside.

Recommended Sample Collection for cNF Trials

To address the research gap in cNF biomarker data, the cNF working group recommends incorporating specimen collection into clinical trials for the study of biomarkers for cNFs. In particular, we recommend collections of uninvolved skin (before and after treatment), cNF (before and after treatment), and plasma (before and after treatment). Collecting uninvolved skin as a comparator tissue is essential for some studies. Careful documentation of the specific characteristics of the cNF and uninvolved skin collected is also a core requirement.

Biomarkers are grouped into 3 major types: protein, RNA, and DNA. Techniques to examine protein biomarkers include immunohistochemistry, Western blot, and nanostring technology (a DNA-barcoded antibody sensing technique that allows multiplexed protein analysis from minimally invasive fine-needle aspirates). Transcriptional (RNA) biomarkers can be measured by quantitative PCR and transcriptome sequencing techniques; these require comparator tissue (uninvolved skin samples) to define differential gene expression. Single-gene (*NF1*) sequencing or somatic tumor analysis can be used to evaluate genetic (DNA) biomarkers.

We recommend the below specific methods for tissue collection with the goal of preserving protein, RNA, and DNA for future analysis because variations in collection or storage of samples may confound results. For serum collection, we suggest PAXgene Blood Tubes for RNA and snap-freezing serum for DNA and protein. For tissue collection, we suggest storage in RNA Later to stabilize RNA, fixation in formalin for DNA and protein, and snap-freezing in liquid nitrogen for DNA and protein.

Furthermore, we need a concerted effort to create standardized phenotypic databases with biobanking capabilities to aid in biomarker discovery. This is especially important when one considers that natural history studies indicate a high degree of

Table 2 Recommended Annotation for cNF Biopsy Samples

Host	Tumor
Germline NF1 mutation	Somatic NF1 mutation
cNF tumor burden	Clinical photographs of the specific lesion(s) sampled
Current age	Tumor subtype (nascent/latent, flat, sessile, globular, and pedunculated)
Sex	Tumor size
Ethnicity	Tumor growth rate
Comorbid conditions	Tumor location
Age at cNF onset	Estimated tumor age

Abbreviations: cNF = cutaneous neurofibroma; NF1 = neurofibromatosis type 1.

disease phenotypic variability within cNFs (even from the same individual) in terms of timing of onset, body location, growth rate, color, maximal size, and related symptoms (pain or itch). Because it is possible that a given biomarker might correlate with one of these characteristics, validating these biomarkers for individual cNF will require careful phenotyping of patients and the individual tumors sampled. Hence, in addition to the fairly extensive recommended clinical dataset suggested by the REiNS Biomarker working group⁴¹ that focuses primarily on the host and includes germline *NF1* mutation, cNF tumor burden (absent, scattered, dense, or unknown), and current age, studies for cNF should also capture disease information such as sex, ethnicity, and age at cNF onset. cNF tissue specimen samples should be documented with size, configuration, consistency, color, growth rate, location on the body from where it was collected, and any associated symptoms specific to that cNF and *NF1* mutation (if known) (table 2). The reason is that it remains unknown whether all cNFs share a common pathogenesis or whether there are important differences between the various forms of cNF seen in people with NF1.⁴² Various schemas to classify cNF have been suggested over the decades, with the most recent working model classifying cNF as nascent/latent, flat, sessile, globular, or pedunculated.

Tradeoffs of Biomarker Trials

There are both advantages and disadvantages to incorporating biomarkers into clinical trials. Biomarkers may enable earlier go/no go decisions for therapeutic trials, saving time and money and improving patient outcomes. In addition, pharmacodynamics biomarkers can provide reassurance that compounds are having the intended on-target effect at tolerable doses. They can also be helpful in dose selection because they can be used to determine bioavailability, target tissue exposure, and therapeutic window. Furthermore, they may be used as inclusion/exclusion criteria for trial enrollment in efficacy studies. Despite these crucial advantages, there are some disadvantages associated with biomarkers. Incorporation of biomarkers into clinical trials can increase the complexity of a study in terms of patient enrollment and

requirements for study support. There is always a danger of overinterpretation of results, and the quality, sensitivity, and reliability of assays are always critical. Use of multiple biomarkers can dramatically increase the complexity of study design and analysis. Infrastructure and experience across clinical trial sites can also impede adequate and timely sample collection. Noncompliance among investigators and study participants (who may not want to have a biopsy or may have wound healing, toxicities, or other skin issue that preclude biopsy) may also create inconsistencies in the trial. Compliance can be complicated because it must be preplanned and real-time infrastructure must be present to confirm that samples are collected in a timely and adequate manner.

Validated systemic and tissue-based biomarkers for cNF would be of great utility for clinical trials, but none have been identified to date. Despite the explosion of NF1-related -omics data, only a handful of studies have used cNF tissues. We recommend incorporation of highly annotated specimen collection into ongoing clinical trials as well as preclinical studies for cNF to identify and validate biomarkers. Progress in this area is likely to support the rapid expansion of therapeutic options for NF1-associated cNF.

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Disclosure

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