Circulating free DNA in the plasma of individuals with neurofibromatosis type 1

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Abstract
Neurofibromatosis type 1 (NF1) is an autosomal dominant syndrome whose characteristic manifestations include benign neurofibromas, yet NF1 is also associated with a high risk of cancer. Measurements of circulating free plasma DNA (cfDNA) are gaining wider applicability in cancer diagnostics, targeting of therapy, and monitoring of therapeutic response. Individuals with NF1 are likely to be followed up using this method, but the effects of NF1 and neurofibromas on cfDNA levels are not known. We studied peripheral blood samples from 19 adults with NF1 and 12 healthy controls. The cfDNA was isolated from plasma with QIAamp Circulating Nucleic Acid Kit and quantified using the Qubit 2.0 Fluorometer. The cfDNA concentration of each sample was normalized relative to the plasma protein concentration. The normalized median concentration of cfDNA in plasma was 19.3 ng/ml (range 6.6–78.6) among individuals with NF1 and 15.9 ng/ml (range 4.8–47.0) among controls (p = .369). Individuals with NF1 who also had plexiform neurofibroma (pNF) showed nonsignificantly elevated cfDNA concentration compared to individuals with NF1 and without known pNF (median 25.4 vs. 18.8 ng/ml, p = .122). The effect of NF1 on cfDNA seems to be relatively small and NF1 is therefore unlikely to hamper the use of cfDNA-based assays.

KEYWORDS
Cancer predisposition syndrome, cancer screening, circulating free DNA, neurofibromatosis type 1, plasma DNA

1 | INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal dominant cancer predisposition syndrome caused by pathogenic variants of the tumor suppressor gene NF1 (Wallace et al., 1990; Gutmann et al., 2017). The prevalence of NF1 is 1/2,000–3,000 (Uusitalo et al., 2015; Kallionpää et al., 2018). NF1 is characterized by benign cutaneous neurofibromas (cNF), café-au-lait macules of the skin, skinfold freckling, Lisch nodules of the eye, skeletal abnormalities, optic pathway gliomas, and plexiform neurofibromas (pNF) (National Institutes of Health Consensus Development Conference, 1988; Gutmann et al., 2017). There are considerable differences in neurofibroma-related tumor burden between individuals with NF1: the number of cNF may vary from less than a dozen to thousands (Plotkin et al., 2012). Although benign, pNF may constitute tumor mass up to several kilograms.

The lifetime risk of cancer in individuals with NF1 is as high as 60%, and the prognosis of NF1-associated cancer is often worse than in the general population (Uusitalo et al., 2016). Tumors of the central...
and peripheral nervous system are characteristic of NF1. pNF may progress to malignant peripheral nerve sheath tumors (MPNSTs) that are associated with a very poor prognosis (Ingham et al., 2011; Gutmann et al., 2017). Unlike the general population, the incidence of MPNSTs starts to increase already at teenage in NF1, and the standardized incidence ratio of MPNST in NF1 may be as high as 2,056 (95% CI 1.561–2.658) (Uusitalo et al., 2016; Peltonen et al., 2019). In addition to tumors of the nervous system and brain, other cancers associated with NF1 include, for example, those of the breast and gastrointestinal tract (Sharif et al., 2007; Uusitalo et al., 2016; Seminog and Goldacre, 2013; Ylä-Outinen et al., 2019; Kenborg et al., 2020).

Measurements based on circulating free DNA (cfDNA) are gaining wider applicability in cancer diagnostics and treatment. Also, non-invasive prenatal testing (NIPT) utilizes cfDNA to analyze fetal DNA from the mother's blood. Due to the increased risk of cancer associated with the NF1 syndrome and the expanding use of NIPT, individuals with NF1 are expected to be frequently subject to cfDNA-based analyses. The cfDNA in overall and the tumor-derived subset of cfDNA, the circulating tumor DNA (ctDNA) in particular may be used to monitor therapeutic response and tumor burden, and to detect residual disease in patients with cancer (Schwarzenbach et al., 2011; Crowley et al., 2013; Heitzer et al., 2015; Ossandon et al., 2018). Moreover, cfDNA analyses, also known as liquid biopsies, can elucidate disease prognosis and allow targeting of therapy when specific actionable alterations are found in ctDNA (Haber and Velculescu, 2014; Bonner et al., 2018). Because cfDNA and ctDNA allow minimally invasive, repeated sampling that is not restricted to a certain region of the tumor like traditional biopsies, they are well suited for, for example, tumors of the nervous system where sampling the tumor may be difficult or carry a high risk for complications (Schwarzenbach et al., 2011; Crowley et al., 2013; Haber and Velculescu, 2014; Heitzer et al., 2015; Bonner et al., 2018; Ossandon et al., 2018; Siena et al., 2018).

A great promise lies in the analysis of cfDNA for early detection of cancers in asymptomatic persons, which would allow treatment at an early and potentially curable stage (Haber and Velculescu, 2014; Amant et al., 2015). While analyses limited to ctDNA allow higher specificity and sensitivity than cfDNA (Crowley et al., 2013), ctDNA analysis is not applicable in asymptomatic individuals where no tumors are initially known. Even if the specificity of cfDNA analysis was not sufficient alone, it can be useful in combination with imaging or protein-based biomarkers such as prostate-specific antigen (Wu et al., 2002; Chun et al., 2006; Schwarzenbach et al., 2011; Heitzer et al., 2015; Bonner et al., 2018). Screening based on cfDNA would be most efficient in persons carrying either genetically or environmentally increased risk for cancer (Haber and Velculescu, 2014), such as the NF1 syndrome.

The plasma cfDNA concentrations among healthy persons typically range between 0 and 100 ng/ml (Jahr et al., 2001; Boddy et al., 2005; Chun et al., 2006; Schwarzenbach et al., 2011; Kim et al., 2014; Devonshire et al., 2014; Fernando et al., 2017; van Ginkel et al., 2017; Kammesheidt et al., 2018). Increased cfDNA concentrations have been reported in, for example, breast cancer (Sunami et al., 2008), sarcoma (Namlos et al., 2017), prostate cancer (Chun et al., 2006), and colorectal cancer (Umetani et al., 2006). However, increases in cfDNA level may also be related to other conditions such as tissue damage and inflammation (Thierry et al., 2014; Schwarzenbach et al., 2011; Heitzer et al., 2015). It is not currently known whether NF1 can affect the individuals’ cfDNA concentration. Such an effect could be possible, for example, because of the benign tumor burden related to cNF and pNF. Knowing how NF1 relates to cfDNA concentration is important for optimizing the use of cfDNA assays in the treatment of cancers occurring in individuals with NF1 as well as when planning screening for NF1-related malignancies. The present study aims at exploring the effects of NF1 on plasma cfDNA to pave the way for use of cfDNA in the diagnosis and treatment of NF1-related complications.

2 | METHODS

2.1 | Editorial policies and ethical considerations

The study was carried out in accordance with the Declaration of Helsinki. It was approved by the Ethics Committee of the Hospital District of Southwest Finland and had study permission from Turku University Hospital. The study was registered in ClinicalTrials.gov with identifier NCT02680431. All participants provided written informed consent for blood sampling and analysis of cfDNA. The individuals with NF1 also consented for the use of their medical records.

2.2 | Participants

Adult patients with NF1 who visited the Turku Neurofibromatosis Centre in Turku University Hospital (Turku, Finland) were eligible for the study. The participant selection was not based on disease manifestations. A total of 21 individuals with NF1, aged 18–64 years, were enrolled in the study. The controls were 14 persons without NF1 or a history of cancer. The controls were frequency matched to individuals with NF1 for age and sex.

2.3 | Sample processing and cfDNA analysis

Peripheral blood was drawn into Li-Heparin tubes and processed within 4 hr of sampling. The plasma cfDNA has previously been reported to be stable for at least 4 hr after sampling (Jung et al., 2003; Lam et al., 2004; Crowley et al., 2013). Blood was diluted 1:1 with phosphate-buffered saline, and plasma was isolated using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation at 2,000g for 30 min. Care was taken to aspirate plasma without disturbing the mononuclear cell layer. The isolated plasma was stored at −80°C until analysis. Upon thawing, 3 ml of plasma was centrifuged at 1,000g for 10 min and the supernatant was collected in order to remove any potential remaining cells. Two samples from
individuals with NF1 and two samples from controls were excluded because of visually detected hemolysis. After exclusion of these samples, all remaining samples showed low absorbance at 414 nm as measured using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

The cfDNA was isolated from 2 ml of plasma using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The cfDNA was eluted into 50 μl of the buffer supplied in the kit. The concentration of double-stranded cfDNA in the eluate was measured in duplicate with Qubit HS Assay Kit (Invitrogen, Eugene, OR) and Qubit 2.0 Fluorometer (Invitrogen) according to the manufacturer’s instructions.

In order to control for variation in plasma dilution during sample processing, protein concentrations of the plasma samples were measured using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL). Each sample was measured at 1:20 and 1:30 dilution and the calculated original concentrations were averaged.

2.4 Clinical information

Clinical information on the history of cancer, optic pathway glioma, and pNF was extracted from the medical records of the patients. The numbers of cNF and subcutaneous neurofibromas (scNF) were estimated by an experienced clinician (SP) at the time of blood sampling.

2.5 Statistical analysis

The protein concentration measured in each sample was divided by the mean of protein concentrations of all samples to obtain a normalization coefficient. The cfDNA concentration values were divided by the normalization coefficient to ensure comparability between samples. For statistical analysis, the normalized cfDNA values were natural logarithm transformed to obtain a Gaussian distribution. Linear regression modeling was used to compare cfDNA values between individuals with NF1 and controls. Both unadjusted, and age-adjusted and sex-adjusted regression models were used. Because of the low number of participants, no multivariate model incorporating both age and sex was constructed. Comparisons by the numbers of cNF and scNF, and history of pNF were conducted within the NF1 group. Two-tailed p values <.05 were considered significant. The R software version 3.3.2 was used for the analyses.

3 RESULTS

Samples from 19 individuals with NF1 and 12 controls were available for analysis (Table 1). The groups were similar in terms of age at sampling and sex. All individuals with NF1 had cNF, more than half of them also had scNF and one third was known to have pNF. Only one individual with NF1 had been diagnosed with optic pathway glioma, and three had a history of cancer 2–19 years before sampling.

The median of unadjusted cfDNA concentration in plasma was 20.0 ng/ml (range 8.2–68.9) among individuals with NF1 and 17.9 ng/ml (range 5.1–38.1) among controls (p = .546). After normalization with respect to plasma protein concentration, the median cfDNA concentration was 19.3 ng/ml (range 6.6–78.6) among individuals with NF1 and 15.9 ng/ml (range 4.8–47.0) among controls (Figure 1). There was no statistically significant difference between individuals with NF1 and controls in the unadjusted analysis (p = .369). Analysis involving both NF1 and sex also indicated no significant effect of NF1 (p = .545, .284) or sex (p = .480, .317) when either the plain or normalized cfDNA concentration was used. However, the analysis adjusted for age at sampling showed a marginally significant difference between individuals with NF1 and controls (p = .079 for plain cfDNA, p = .023 for normalized cfDNA), and also the interaction between NF1 and age at sampling was marginally significant (p = .085 for plain cfDNA, p = .032 for normalized cfDNA). As a sensitivity analysis, we excluded one individual with NF1 with a much higher cfDNA concentration than observed in the others (Figure 1), yet the difference in normalized cfDNA concentration

| TABLE 1 Characteristics of the individuals with NF1 and controls included in the cfDNA analysis |
|-------------------------------------------------|-------------------------------------------------|
| Individuals with NF1 | Controls |
| n | 19 | 12 |
| Age (years; mean, SD) | 36.0 (12.9) | 38.7 (12.0) |
| Sex | | |
| Women (n, %) | 11 (57.9%) | 5 (41.7%) |
| Men (n, %) | 8 (42.1%) | 7 (58.3%) |
| Cutaneous neurofibromas (n, %) | | |
| None | 0 (0.0%) | | |
| 1–5 | 1 (5.3%) | | |
| 6–49 | 12 (63.2%) | | |
| 50–99 | 3 (15.8%) | | |
| 100–500 | 2 (10.5%) | | |
| >500 | 1 (5.3%) | | |
| Subcutaneous neurofibromas (n, %) | | |
| None | 8 (42.1%) | | |
| 1 | 0 (0.0%) | | |
| 2–5 | 5 (26.3%) | | |
| 6–10 | 1 (5.3%) | | |
| >10 | 3 (15.8%) | | |
| Not available | 2 (10.5%) | | |
| Plexiform neurofibroma (n, %) | | |
| Yes | 6 (31.6%) | | |
| None known | 13 (68.4%) | | |
between individuals with NF1 and controls persisted in the age-adjusted analysis ($p = .045$). Among individuals with NF1 only, older individuals had lower cfDNA concentration (plain cfDNA, Spearman’s $\rho = -0.72$; normalized cfDNA, Spearman’s $\rho = -0.55$). No pattern between the normalized cfDNA concentration and the number of cNF was observed among individuals with NF1 (Figure 2). The results were essentially the same when plain cfDNA concentration without normalization was used (data not shown). Individuals with six or more scNF seemed to have higher normalized cfDNA concentration than individuals with <6 scNF (Figure 2), yet the difference was not statistically significant ($p = .514$). Moreover, the median of normalized plasma cfDNA concentration was 25.4 ng/ml (range 10.7–78.6) among individuals with NF1 and pNF, and 18.8 ng/ml (range 6.6–34.6) among individuals with NF1 without pNF ($p = .122$). The individual with the highest concentration of cfDNA was known to have two internal pNF and at least one atypical neurofibroma.

**FIGURE 1** Normalized plasma concentrations of circulating free DNA (cfDNA) in individuals with neurofibromatosis type 1 (NF1) and controls. Age, sex, the estimated numbers of cutaneous neurofibromas (cNF) and subcutaneous neurofibromas (scNF), and history of plexiform neurofibroma (pNF) are shown for each person.

### DISCUSSION

No significant difference between individuals with NF1 and controls was observed in the unadjusted analysis. Older age was associated with lower cfDNA concentration among individuals with NF1 but not among controls, which explains why the difference between the two groups was statistically marginally significant after adjustment for age. Even then, the observed difference between individuals with NF1 and controls was relatively small and it is likely not clinically significant. Our data suggest that pNF and scNF may be related to higher cfDNA concentration among individuals with NF1, yet the current sample size and the lack of volumetric information on tumor burden do not allow us to prove such an association. The number of cNF as such does not seem to associate with the concentration of cfDNA.

Since the change in cfDNA concentration caused by the NF1 syndrome and neurofibromas is small, NF1 is not expected to reduce the sensitivity of cfDNA-based assays. In NIPT, the ratio of fetal to maternal cfDNA affects the sensitivity of the assay (Wang et al., 2013). Moreover, a wide variation in the proportion of ctDNA out of total cfDNA has been observed in studies of patients with tumors (Jahr et al., 2001; Heitzer et al., 2015). It is thus essential to know how NF1 and the associated benign tumor burden affect cfDNA concentration to optimize assays used for individuals with NF1. Although further studies are needed, the analysis of cfDNA holds a great promise for individuals with NF1 who are at a highly increased risk for various malignancies, often associated with a poor prognosis (Ususitalo et al., 2016). Early detection of the tumors might improve the prognosis of NF1-associated cancers. The analysis of cfDNA could prove particularly useful in cases where detecting the transformation of a benign pNF into MPNST poses a challenge. The tumor may be highly heterogeneous and invasive procedures carry a high risk of complications related to neural and vascular damage, highlighting the need for a minimally invasive sampling procedure, such as liquid biopsy (Bonner et al., 2018).

The characteristic tumors of NF1, benign neurofibromas, may form a substantial tumor burden and it is thus plausible that they could modify cfDNA concentrations. NF1-related tumors are also known to contain immune cells (Karmakar and Reilly, 2017), suggesting a potential inflammatory process, which could affect cfDNA release. It is possible that age changes the intratumoral inflammation, which affects the cfDNA concentrations in NF1. Our observation of a declining cfDNA concentration by increasing age is in contrast to previous studies suggesting an association of older age with higher cfDNA levels in some groups (Kim et al., 2014; Meddeb et al., 2019) and may thus reflect a process specific to NF1. It can also be hypothesized that the older individuals with NF1 have mild disease manifestations, as severe complications would have led to an early death. However, the upper quartile of age at sampling was 42.5 years among participants with NF1, suggesting that bias related to the severity of NF1 manifestations and premature mortality is not likely to be significant.

We quantified the cfDNA using a fluorescence-based assay. Many previous studies have used PCR-based methods for cfDNA
quantification (Umetani et al., 2006; Sunami et al., 2008; Devonshire et al., 2014). However, fluorescence-based quantification has good sensitivity (Wu et al., 2002), is easily applicable in clinical settings (Tissot et al., 2015) and also allows the quantification of non-amplifiable cfDNA (Jahr et al., 2001; Szpechcinski et al., 2008). Fluorescence-based cfDNA assays have previously shown high correlation with PCR-based methods (Szpechcinski et al., 2008; Fernando et al., 2017). Since there are no mutational hotspots in the NF1 gene (Koczkowska et al., 2020) and no other genes are known to be recurrently mutated in neurofibromas, measuring ctDNA specifically was not feasible in the setting of the present study. The cfDNA values were normalized relative to plasma protein concentration to take into account any variation during sample processing. Theoretically, the normalization could obscure the results if there was significant physiological variation of plasma protein concentration unrelated to the concentration of cfDNA. However, our analyses based on the plain cfDNA concentration were highly concordant with those using normalized data, indicating the robustness of the findings.

The median cfDNA concentrations of 20 and 18 ng/ml among individuals with and without NF1, respectively, are higher compared to studies reporting cfDNA levels <10 ng/ml in the healthy population (Boddy et al., 2005; Devonshire et al., 2014; Fernando et al., 2017). On the other hand, concentrations up to 100 ng/ml have been regularly reported in healthy populations (Chun et al., 2006; Schwarzenbach et al., 2011; Kim et al., 2014; van Ginkel et al., 2017; Kammesheidt et al., 2018). The varying estimates are most likely due to methodological differences between studies. Even if the methods utilized here yield higher cfDNA concentrations than in some previous studies, the within-study comparison between individuals with and without NF1 remains valid. The number of individuals included in the current explorative study is very limited. Moreover, the cfDNA concentrations were only compared to the clinical features observed at the time of blood sampling. The participants were not screened for pNF and indolent tumors may not have been detected. Further studies are needed to establish the effects of NF1 on cfDNA in larger samples and to assess the potential of cfDNA to predict NF1-related complications before clinical symptoms. In order to definitely determine whether the cfDNA from benign NF1-related tumors affects the signal from malignant tumors, individuals with cancer and either low or high benign tumor burden should be studied, preferably repeatedly over time, and the volumetric tumor burden should be correlated with cfDNA and ctDNA levels. Naturally, a similar setting could be employed during pregnancy to assess the utility of NIPT.

The current results show that NF1 may affect the plasma cfDNA concentration, but the effect is relatively small. Further studies are needed to elucidate the contribution of pNF on cfDNA, yet based on the present results, NF1 is not likely to hamper cfDNA-based assays.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
Rooke A. Kallionpää, Juha Peltonen, and Sirkku Peltonen designed the study. Sirkku Peltonen collected the blood samples and clinical information. Roope A. Kallionpää, Kaisa Ahramo, Marianna Aaltonen, and Paula Pennanen performed the laboratory analyses. Roope
A. Kallionpää analyzed the data and drafted the manuscript. All authors revised the manuscript and approved its submission for publication.

**DATA AVAILABILITY STATEMENT**

The data of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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**REFERENCES**

Amant, F., Verheeecke, M., Wlodarska, I., Dehaspe, L., Brady, P., Brison, N., ... Vermeesch, J. R. (2015). Presymptomatic identification of cancers in pregnant women during noninvasive prenatal testing. *JAMA Oncology*, 1, 814–819.

Boddy, J. L., Gal, S., Malone, P. R., Harris, A. L., & Wainscoat, J. S. (2005). Prospective study of quantitation of plasma DNA levels in the diagnosis of malignant versus benign prostate disease. *Clinical Cancer Research*, 11, 1359–1399.

Bonner, E. R., Bornhorst, M., Packer, R. J., & Nazarian, J. (2018). Liquid biopsy for pediatric central nervous system tumors. *NPJ Precision Oncology*, 2, 29.

Chun, F. K.-H., Müller, I., Lange, I., Friedrich, M. G., Erbersdobler, A., Karakiewicz, P. I., ... Schwarzenbach, H. (2006). Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. *BJU International*, 98, 544–548.

Crowley, E., Di Nicolantonio, F., Loupakis, F., & Bardelli, A. (2013). Liquid biopsy: Monitoring cancer-genetics in the blood. *Nature Reviews Clinical Oncology*, 10, 472–484.

Devonshire, A. S., Whale, A. S., Gutteridge, A., Jones, G., Cowen, S., Foyston, C. A., & Huggett, J. F. (2014). Towards standardisation of cell-free DNA measurement in plasma: Controls for extraction efficiency, fragment size bias and quantification. *Analytical and Bioanalytical Chemistry*, 406, 6499–6512.

Fernando, M. R., Jiang, C., Krzyzanowski, G. D., & Ryan, W. L. (2017). New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. *PLoS One*, 12, e0183915.

van Ginkel, J. H., van den Broek, D. A., van Kuik, J., Linders, D., de Weger, R., Willems, S. M., & Hubers, M. M. H. (2017). Preanalytical blood sample workup for cell-free DNA analysis using droplet digital PCR for future molecular cancer diagnostics. *Cancer Medicine*, 6, 2297–2307.

Gutmann, D. H., Ferner, R. E., Listerick, R. H., Korf, B. R., Wolters, P. L., & Johnson, K. J. (2017). Neurofibromatosis type 1. *Nature Reviews Disease Primers*, 3, 17004.

Harber, D. A., & Velculescu, V. E. (2014). Blood-based analyses of cancer: Circulating tumor cells and circulating tumor DNA. *Cancer Discovery*, 4, 650–661.

Heitzer, E., Ulz, P., & Geigl, J. B. (2015). Circulating tumor DNA as a liquid biopsy for cancer. *Clinical Chemistry*, 61, 112–123.

Ingham, S., Huson, S. M., Moran, A., Wylie, J., Leahy, M., & Evans, D. G. R. (2011). Malignant peripheral nerve sheath tumours in NF1: Improved survival in women and in recent years. *European Journal of Cancer*, 47, 2723–2728.

Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D., & Knippers, R. (2001). DNA fragments in the blood plasma of cancer patients: Quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Research*, 61, 1659–1665.

Jung, M., Klotzke, S., Lewandowski, M., Fleischhacker, M., & Jung, K. (2003). Changes in concentration of DNA in serum and plasma during storage of blood samples. *Clinical Chemistry*, 49, 1028–1029.

Kallionpää, R. A., Uusitalo, E., Leppävirta, J., Pöyhönen, M., Peltonen, S., & Peltonen, J. (2018). Prevalence of neurofibromatosis type 1 in the Finnish population. *Genetics in Medicine*, 20, 1082–1086.

Kammesheidt, A., Tonozzi, T. R., Lim, S. W., & Braunstein, G. D. (2018). Mutation detection using plasma circulating tumor DNA (ctDNA) in a cohort of asymptomatic adults at increased risk for cancer. *International Journal of Molecular Epidemiology and Genetics*, 9, 1–12.

Karmakar, S., & Reilly, K. M. (2017). The role of the immune system in neurofibromatosis type 1 associated nervous system tumors. *CNS Oncology*, 6, 45–60.

Kenborg, L., Duun-Henriksen, A. K., Dalton, S. O., Bidstrup, P. E., Doser, K., Rugbjerg, K., ... Winther, J. F. (2020). Multisystem burden of neurofibromatosis 1 in Denmark: Registry- and population-based rates of hospitalizations over the life span. *Genetics in Medicine*, 22, 1069–1078.

Kim, K., Shin, D. G., Park, M. K., Baik, S. H., Kim, T. H., Kim, S., & Lee, S. (2014). Circulating cell-free DNA as a promising biomarker in patients with gastric cancer: Diagnostic validity and significant reduction of ctDNA after surgical resection. *Annals of Surgical Treatment and Research*, 86, 136–142.

Koczkwoska, M., Callens, T., Chen, Y., Gomes, A., Hicks, A. D., Sharp, A., ... Messiaen, L. M. (2020). Clinical spectrum of individuals with pathogenic NF1 missense variants affecting p.Met1149, p.Arg1276, and p.Lys1423. Genotype–phenotype study in neurofibromatosis type 1. *Human Mutation*, 41, 299–315.

Lam, N. Y. L., Rainer, T. H., Chiu, R. W. K., & Lo, Y. M. D. (2004). EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. *Clinical Chemistry*, 50, 256–257.

Meddeb, R., Dache, Z. A. A., Thezenas, S., Otandault, A., Tanos, R., Pastor, B., ... Thierry, A. R. (2019). Quantifying circulating cell-free DNA in humans. *Scientific Reports*, 9, 5220.

Namlas, H. M., Zaikova, O., Bjerkehagen, B., Vodák, D., Hovig, E., Myklebost, O., ... Meza-Zepeda, L. A. (2017). Use of liquid biopsies to monitor disease progression in a sarcoma patient: A case report. *BMC Cancer*, 17, 29.

National Institutes of Health Consensus Development Conference. (1988). *Neurofibromatosis*. Conference statement. *Archives of Neurology*, 45, 575–578.

Ossandon, M. R., Agrawal, L., Bernhard, E. J., Conley, B. A., Dey, S. M., Divi, R. L., ... Tricoli, J. V. (2018). Circulating tumor DNA assays in clinical cancer research. *Journal of the National Cancer Institute*, 110, 929–934.

Peltonen, S., Kallionpää, R. A., Rantanen, M., Uusitalo, E., Lähteenmäki, P. M., Pöyhönen, M., ... Peltonen, J. (2019). Pediatric malignancies in neurofibromatosis type 1: A population-based cohort study. *International Journal of Cancer*, 145, 2926–2932.

Plotkin, S. R., Bredella, M. A., Cai, W., Kassarjian, A., Harris, G. J., Espanar, S., ... Mautner, V. F. (2012). Quantitative assessment of whole-body tumor burden in adult patients with neurofibromatosis. *PLoS One*, 7, e35711.

Schwarzenbach, H., Hoon, D. S. B., & Pantel, K. (2011). Cell-free nucleic acids as biomarkers in cancer patients. *Nature Reviews Cancer*, 11, 426–437.

Semigov, O. O., & Goldacre, M. J. (2013). Risk of benign tumours of nervous system and of malignant neoplasms, in people with neurofibromatosis: *Population-based record-linkage study*. *British Journal of Cancer*, 108, 193–198.

Sharif, S., Moran, A., Huson, S. M., Iddenden, R., Shenton, A., Howard, E., ... Evans, D. G. R. (2007). Women with neurofibromatosis 1 are at a moderately increased risk of developing breast cancer and should be considered for early screening. *Journal of Medical Genetics*, 44, 481–484.
Siena, S., Sartore-Bianchi, A., Garcia-Carbonero, R., Karthaus, M., Smith, D., Tabernero, J., ... Bardelli, A. (2018). Dynamic molecular analysis and clinical correlates of tumor evolution within a phase II trial of panitumumab-based therapy in metastatic colorectal cancer. *Annals of Oncology: Official Journal of the European Society for Medical Oncology*, 29, 119–126.

Sunami, E., Vu, A. T., Nguyen, S. L., Giuliano, A. E., & Hoon, D. S. B. (2008). Quantification of LINE1 in circulating DNA as a molecular biomarker of breast cancer. *Annals of the New York Academy of Sciences*, 1137, 171–174.

Szpechcinski, A., Struniawski, R., Zaleska, J., Chabowski, M., Orłowski, T., Roszkowski, K., & Chorostowska-Wynimko, J. (2008). Evaluation of fluorescence-based methods for total vs. amplifiable DNA quantification in plasma of lung cancer patients. *Journal of Physiology and Pharmacology*, 59, 675–681.

Thierry, A. R., Mouliere, F., El Messaoudi, S., Mollevi, C., Lopez-Crapez, E., Rolet, F., ... Ychou, M. (2014). Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. *Nature Medicine*, 20, 430–435.

Tissot, C., Toffart, A.-C., Villar, S., Souquet, P.-J., Merle, P., Morosibilot, D., ... Couraud, S. (2015). Circulating free DNA concentration is an independent prognostic biomarker in lung cancer. *The European Respiratory Journal*, 46, 1773–1780.

Umetani, N., Kim, J., Hiramatsu, S., Reber, H. A., Hines, O. J., Blichik, A. J., & Hoon, D. S. B. (2006). Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: Direct quantitative PCR for ALU repeats. *Clinical Chemistry*, 52, 1062–1069.

Uusitalo, E., Leppävirta, J., Koffert, A., Suominen, S., Vahtera, J., Vahlberg, T., ... Peltonen, S. (2015). Incidence and mortality of neurofibromatosis: A total population study in Finland. *Journal of Investigative Dermatology*, 135, 904–906.

Uusitalo, E., Rantanen, M., Kallionpää, R. A., Pöyhönen, M., Leppävirta, J., Ylä-Outinen, H., ... Peltonen, J. (2016). Distinctive cancer associations in patients with neurofibromatosis type 1. *Journal of Clinical Oncology*, 34, 1978–1986.

Wallace, M. R., Marchuk, D. A., Andersen, L. B., Letcher, R., Odeh, H. M., Saulino, A. M., ... Collins, F. S. (1990). Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. *Science*, 249, 181–186.

Wang, E., Batey, A., Strubie, C., Musci, T., Song, K., & Oliphant, A. (2013). Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. *Prenatal Diagnosis*, 33, 662–666.

Wu, T. L., Zhang, D., Chia, J. H., Tsao, K. C., Sun, C. F., & Wu, J. T. (2002). Cell-free DNA: Measurement in various carcinomas and establishment of normal reference range. *Clinica Chimica Acta*, 321, 77–87.

Ylä-Outinen, H., Loponen, N., Kallionpää, R. A., Peltonen, S., & Peltonen, J. (2019). Intestinal tumors in neurofibromatosis 1 with special reference to fatal gastrointestinal stromal tumors (GIST). *Molecular Genetics & Genomic Medicine*, 7, e927.

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