IGF2 is a potential factor in RAI-refractory differentiated thyroid cancer

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Abstract. Differentiated thyroid cancer (DTC) is the most frequent endocrine tumor with a good prognosis after primary treatment in most cases. By contrast, 30–40% of patients with metastatic DTC are unresponsive to 131I radioactive iodide (RAI) treatment due to tumor dedifferentiation. Currently, underlying molecular mechanisms of dedifferentiation remain elusive and predictive biomarkers are lacking. Therefore, the present study aimed to identify molecular biomarkers in primary tumors associated with RAI refractoriness. A retrospective cohort was gathered consisting of RAI-sensitive patients with DTC and RAI-refractory patients with poorly DTC. In all patients, extensive intratumoral mutation profiling, gene fusion analysis, telomerase reverse transcriptase (TERT) promoter mutation analysis and formalin-fixed paraffin-embedded compatible RNA sequencing were performed. Genetic analyses revealed an increased mutational load in RAI-refractory DTC, including mutations in AKT1, PTEN, TP53 and TERT promoter. Transcriptomic analyses revealed profound differential expression of insulin-like growth factor 2 (IGF2), with up to 100-fold higher expression in RAI-refractory DTC compared with in RAI-sensitive DTC cases. ELISA revealed significant lower IGF2 plasma concentrations after surgery and subsequent 131I RAI therapy in patients with DTC compared with pretreatment baseline. Overall, the current findings suggested that the tumor-promoting growth factor IGF2 may have a potential role in acquiring RAI refractoriness.

Introduction

Differentiated thyroid cancer (DTC) is the most frequent endocrine tumor with in most cases a good prognosis. Standard of care consists of hemi-thyroidectomy or total thyroidectomy in acquiring RAI refractoriness. The same holds true for the assessment of sodium-iodide symporter expression in circulating tumor cells (10). Also, prostate-specific membrane antigen (PSMA) expression in tumor tissue has been suggested to contribute in the prediction of tumor aggressiveness and patient outcome (11). The most intensively studied marker is Thyroglobulin (Tg). Quantitative changes in Tg could reflect the response to RAI therapy or serve as diagnostic or prognostic tool (12-14). However, current methods, such as serum Tg or PSMA expression measurements serve mostly as a diagnostic, predictive or prognostic tool. Therefore, the identification of DTC patients at risk for RAI refractory disease is still an unmet medical need and additional markers need to be explored.

In the present study we aimed to identify molecular markers in primary tumors that are associated with RAI
refractory disease. A retrospective cohort of 63 DTC patients, including all histological subtypes, was collected for this study and consisted of 35 RAI-sensitive DTC patients and 28 RAI-refractory (poorly) differentiated TC patients. Extensive intratumoral mutation profiling, gene fusions analysis, \textit{TERT} promoter mutation analysis and formalin-fixed paraffin embedded-compatible RNA sequencing was performed in all patients. To validate potential circulating markers, an independent cohort of 8 DTC patients was available.

**Materials and methods**

**Patient cohorts.** Detailed pathology reports were collected of all DTC patients diagnosed in Nijmegen and surrounding hospitals between 2000 and 2016 (1,544 patients). We selected 35 RAI-sensitive DTC patients, ages ranged from 15 to 77 years (M=41.9±18.9), and 28 RAI-refractory DTC patients, ages ranged from 45 to 84 years (M=61.9±10.2), that underwent total or near-total thyroidectomy and showed residual disease after primary surgery. The eighth edition of the American Joint Committee on Cancer (AJCC) staging system was used to determine the TNM stage of each individual patient (15). Patients with confirmed nodal metastases prior to primary surgery also underwent a modified radical lymph node dissection. RAI ablation of residual thyroid tissue was performed 4-6 weeks after surgery. All patients included in this study had residual disease after primary surgery as demonstrated by diagnostic RAI scintigraphy. If indicated, patients were repeatedly treated with RAI to reach remission. RAI sensitivity was defined as a complete response to RAI therapy of histologically differentiated tumor lesions resulting in remission after the primary treatment by surgery and RAI ablation or (if indicated) after subsequent treatments with RAI for metastases with documented $^{131}$I uptake. Remission was defined as undetectable thyroid-stimulating hormone stimulated Tg in the absence of anti-Tg antibodies and no evidence of loco-regional disease or distant metastasis on the whole-body iodine scans (WBS) and/or neck ultrasonographic examinations at 6-9 months after the last RAI treatment. The remission status was confirmed at the last follow-up visit. According to the RECIST criteria, RAI refractoriness was defined as either new evidence of recurrent loco-regional disease or distant metastasis after successful primary therapy or progressive disease at least 6 months after primary treatment by surgery and RAI treatment preferably supported by presence of metastases that do not accumulate $^{131}$I on the last post-therapy scan. Persistent disease was defined as detectable Tg and/or evidence of loco-regional disease or distant metastases. Of all selected DTC patients, archived formalin-fixed paraffin-embedded (FFPE) tissue specimens were collected for genetic, transcriptomic and protein analyses. Collection, storage and use of archival tissue and patient data were in compliance with the ‘Code of Proper Secondary Use of Human Tissue in the Netherlands’ (http://www.fmvw.nl and www.federa.org). This study was approved by the Research Ethics Committee [Commissie Mensgebonden Onderzoek (CMO)] of the Radboud University Medical Center under application 2015-1762 and followed the ethical guidelines of the CMO. The CMO waived the need for consent for the use of archive samples since the samples were analyzed anonymously.

An independent cohort including eight consecutive newly diagnosed patients with DTC (with and without metastases), ages ranged from 27 to 78 years (M=54.6±16.5), that were therapy naïve and were planned to receive conventional primary therapy by surgery followed by RAI were included in which plasma insulin-like growth factor 2 (IGF2) concentrations were measured before surgery and 30 days after $^{131}$I radioactive iodide therapy. This study was also approved by the Research Ethics Committee [Commissie Mensgebonden Onderzoek (CMO)] of the Radboud University Medical Center under application: 2017-3628; NL62671.091.17; ClinicalTrials.gov Identifier: NCT03397238. Informed consent was obtained from all participants and/or their legal guardians. Their results were compared to those of six gender and aged matched healthy volunteers.

**DNA and RNA isolation from FFPE tissues.** For DNA isolation, 60 µm FFPE tissue slices were digested overnight at 56°C in the presence of TET-lysis buffer (10 mM Tris/HCl pH 8.5, 1 mM EDTA pH 8.0, 0.01% Tween-20), 5% Chelex-100 (Bio-Rad Laboratories, Inc.), 15 µg/ml GlycoBlue (Life Technologies) and 400 µg proteinase K (Qiagen), followed by inactivation at 95°C for 10 min. For DNA isolation, the supernatant was transferred after centrifugation (20817 rcf), cooled on ice and precipitated in the presence of 100% EtOH and 1/33 volume 3 M NaAc (pH 5.2). Pellets were washed with cold 70% EtOH, dissolved in 80 µl Tris-EDTA and DNA concentrations were determined using the Qubit Broad Range kit (Thermo Fisher Scientific, Inc.). For RNA isolation, 60 µm FFPE tissue slices were digested overnight at 56°C in the presence of 240 µl lysis buffer (Qiagen) and 400 µg proteinase K. Next, supernatants were transferred after centrifugation (16,000 x g) and mixed with RNA-Bee. Subsequently, RNA was isolated by phase separation with chloroform and precipitated by isopropanol, according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.).

**Intratumoral mutation profiling.** Somatic mutations in human DTC tumor tissue were detected by our in-house Cancer Hotspot Panel based on single-molecule molecular inversion probes, as described previously (16). Isolated DNA from FFPE tissues with a tumor cell percentage of $>60%$ was subjected to library preparation and clinically relevant regions were sequenced of the following genes: AKT1, BIRC3, BRAF, CHEK2, CTPNB1, CXCR4, EGFR, ERBB2, EZH2, GNA11, GNAQ, GNAS, H3F3A, H3F3B, HRAS, IDH1, IDH2, JAK2, KIT, KRAS, MPL, MS1H2, MYD88, NRAS, PDGFR, PIK3CA, SF3B1, SLC7A8 and ZNF2.

**Gene fusion analysis.** DNA/RNA was isolated from punched tumor tissue with a tumor cell percentage of $>50%$. Gene fusion analysis was performed by Next Generation Sequencing (Archer FusionPlex CTL Panel) and data were analyzed by Archer Analysis software (version 5). Relevant fusions of the following target genes were sequenced: ALK (5'; exons 2, 4, 6, 10, 16-23, intron 19), AXL (3'; exons 18-20, BRAF (5'; exons 7-11, 3'); exons 7, 8, 10), CCND1 (5'; exons 1-4, 3'; exons 1, 2, 4), FGFFR1 (5'; exons 2, 8-10, 17, 3'; exon 17), FGFR2 (5'; exons 2, 5, 7-10, 3'; exon 17), FGFR3 (5'; exons 3, 5, 8-10, 3'; exon 17, intron 17), MET (5'; exons 2, 4-6, 13, 14, 16,
DTC patients show significantly lower circulating levels of IGF2 concentration after primary treatment. To further support the role of IGF2 in the pathogenesis of DTC we investigated the IGF2 plasma concentration before and after treatment in an independent cohort, including eight newly diagnosed therapy-naive patients with DTC (4 males and 4 females, aged 54.6±16.5 years) scheduled for conventional treatment by surgery followed by RAI. Patients and tumor characteristics of this cohort are listed in Table III. The IGF2 plasma concentrations in these patients were compared to those of six age- and gender-matched healthy volunteers (3 males and 3 females, averaged 51.7±12.4 years). The average total IGF2 concentration before surgery (baseline) in DTC patients showed higher (1.17±10^5/+/−1.17±10^5 pg/ml) compared to healthy volunteers (9.5±10^5/+/−1.7±10^5 pg/ml) although this difference did not reach statistical significance (Fig. 2A). Thirty days after primary surgery the average IGF2 plasma concentration was significantly decreased (8.3±10^5/+/−2.6±10^5 pg/ml) compared to the level before surgery (Fig. 2B). To exclude interference between IGF2 and its ligand IGF2 effecting the assay, we assessed the plasma IGF2 concentrations in the same samples. The IGFBP2 concentrations after therapy were not significantly different from those measured before surgery, which suggests an even stronger effect of treatment on the free IGF2 circulating concentrations (Fig. 2C).

Discussion

Obliterating tumor remnants after thyroidectomy by RAI therapy is of significant clinical importance in DTC. Unresponsiveness to RAI is a major concern since RAI-refractory tumors usually respond poorly to alternative therapies. Apart from a lack of therapeutic options,
markers predicting RAI refractoriness have so far not been identified. Therefore, we searched for differences between the molecular signatures obtained from primary RAI sensitive and RAI refractory DTC samples. Genetic analyses revealed an increased mutational load in RAI-refractory DTC, including mutations in \textit{AKT1}, \textit{PTEN}, \textit{TP53} and \textit{TERT} promoter. Mutations or deletions of the tumor suppressor gene \textit{PTEN} are genetic alterations that can activate the PI3K-Akt pathway (1). Other genetic alterations activating the PI3K-Akt pathway in DTC tumors, particularly in those having a poor prognosis, include mutations encoding for \textit{PI3KCA}, \textit{AKT1} and \textit{RAS} genes. As genetic alterations accumulate, additional mutations occur in genes such as \textit{TP53}. Additional mutations, together with the initial genetic alterations in the MAPK or PI3K-Akt pathway contribute to tumor progression and could ultimately lead to the development of PDTC or anaplastic thyroid cancer (ATC) (1,17). Previous studies have also shown that somatic \textit{TERT} promoter mutations are more prevalent in aggressive types of TC such as PDTC and ATC (18,19). Our observations, demonstrating a higher proportion of tumors bearing \textit{TERT} promoter mutations in RAI-refractory DTC patients, are in concordance with these studies. Interestingly, transcriptome data from RAI refractory DTC patients and RAI sensitive DTC patients revealed a significantly higher RNA expression of \textit{IGF2} in primary tumors of RAI refractory DTC patients. Moreover, we show that the IGF2 plasma concentration in patients with DTC significantly decreased after primary treatment by surgery and RAI. Our data suggest no clear relationship between the increased mutational load and the overexpression of \textit{IGF2} in RAI-refractory DTC patients. However, due to the relatively small sample size our study probably lacked the statistical power to robustly assess this association. Therefore, the relationship between the increased mutational load and overexpression of \textit{IGF2} should

| Parameter                        | RAI-sensitive DTC | RAI-refractory DTC | P-value |
|----------------------------------|-------------------|--------------------|---------|
| Mean age at diagnosis ± SD, years| 41.9±18.9         | 61.9±10.2          | <0.001  |
| Sex, male/female, n              | 13/22             | 16/12              | 0.134   |
| Histology, n                     |                   |                    | 0.0045  |
| PTC                              | 29                | 11                 |         |
| FTC                              | 4                 | 10                 |         |
| HCC                              | 1                 | 5                  |         |
| FVPTC                            | 1                 | 2                  |         |
| T-stage, n                       |                   |                    | 0.0011  |
| T1                               | 12                | 3                  |         |
| T2                               | 12                | 4                  |         |
| T3                               | 8                 | 7                  |         |
| T4                               | 3                 | 14                 |         |
| N-stage, n                       |                   |                    | NA      |
| N0                               | 0                 | 0                  |         |
| N1                               | 35                | 28                 |         |
| M-stage, n                       |                   |                    | <0.0001 |
| M0                               | 31                | 4                  |         |
| M1                               | 4                 | 24                 |         |
| Location of metastases, n        |                   |                    | <0.0001 |
| No metastases                    | 31                | 4                  |         |
| Lung                             | 3                 | 16                 |         |
| Bone                             | 0                 | 2                  |         |
| Lung and bone                    | 1                 | 5                  |         |
| Lung, liver and muscle           | 0                 | 1                  |         |
| Number of RAI treatments, n      |                   |                    | 0.0032  |
| 0-1                              | 22                | 6                  |         |
| 2                                | 9                 | 12                 |         |
| >2                               | 4                 | 10                 |         |
| Mean cumulative RAI dose ± SD, MBq| 9,806±7,127       | 17,240±8,955       | <0.001  |

*Based on AJCC 8th edition of TNM classification. Data analyzed by Student’s t-test or Fisher’s exact test. AJCC, American Joint Committee on Cancer; DTC, differentiated thyroid cancer; FTC, follicular thyroid cancer; FVPTC, follicular-variant papillary thyroid cancer; HCC, Hürthle cell carcinoma; PTC, papillary thyroid cancer; RAI, radioactive iodide; NA, not applicable.
be investigated in future studies including larger cohorts of patients.

The IGFR2 gene is located on chromosome 11p15.5 and encodes for the IGFR2 growth factor that has been shown to play an important role in the fetal embryonic development, growth and energy metabolism of mammals (20‑24). Apart from this, IGFR2 has also been reported to be involved in carcinogenesis. In several cancer types, increased expression of IGFR2 has been linked to poor prognosis (25). In lung cancer patients, increased IGFR2 protein expression in pleural effusion supernatants was associated with resistance to osimertinib treatment (26). Elevated levels of IGFR2 mRNA in osteosarcoma cells were shown after chemotherapy resulting in preservation of these cells under chemotherapeutic stress (27). In a study investigating 445 gastrointestinal tumors, high protein expression of IGFR2 in the tumor tissue was associated with a significant worse outcome (28). In colorectal cancer, overexpression of stromal‑derived IGFR2 has been shown to play a role in development and progression, whereas increased serum concentrations and tissue overexpression of IGFR2 has been associated with metastasis (29‑31). Moreover, in breast cancer tissues, IGFR2 was found to be more potent than in normal breast tissue for activating insulin receptor (IR) autophosphorylation causing stimulation of cell growth (32). Finally, a study performed by Tominaga et al described a positive feedback loop, IGFR2‑IGF1R‑PI3K‑ID1‑IGFR2, present in cancer stem‑like cells causing cells to maintain in the stem cell state (33).}

A few other studies suggest a role for IGFR2 in the pathogenesis of DTC. Differences in IGFR2 mRNA expression between normal thyroid epithelial cells, thyroid adenoma and thyroid carcinoma were demonstrated using three pairwise comparisons with the GEO2R online tool (34). One research group published several studies in which they

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**Table II. Genetic characteristics of RAI‑sensitive (n=35) and RAI‑refractory (n=28) patients with DTC.**

| Parameter                  | RAI‑sensitive DTC, n | RAI‑refractory DTC, n | P‑value |
|----------------------------|----------------------|----------------------|---------|
| Oncogenic mutation status  |                      |                      | 0.442   |
| BRAF V600E                 | 15                   | 9                    |         |
| H/K/NRAS, G12D/Q61R        | 4                    | 5                    |         |
| Other                      |                      |                      |         |
| CCDC6:RET fusion (n=4)     |                      | PTEN E242X and TP53 R158G |
| CCDC6:RET fusion and PIK3CA |                      | (n=1), PTEN E242X and TP53 P212fs (n=1), NRAS Q61R and |
| H1047R (n=1), SQSTM1:NTRK3  |                      | AKT1 E17L (n=1),     |
| fusion (n=1), ETV6:NTRK3 fusion (n=1), NCOA4:RET fusion (n=1), |                      | EML4:NTRK3 fusion (n=1) |
| PAX8:PPARG fusion (n=2)    |                      |                      |         |
| Unknown                    | 6                    | 10                   | 0.0004  |
| TERT promoter mutation status |                    |                      |         |
| TERT wild‑type             | 32                   | 14                   |         |
| TERT C228T                 | 3                    | 14                   |         |

Data analyzed by Fisher's exact test. DTC, differentiated thyroid cancer; RAI, radioactive iodide; TERT, telomerase reverse transcriptase.
Table III. Clinical characteristics of patients with differentiated thyroid cancer from the independent cohort.

| Patient no. | Age at inclusion, years | Sex | Histology | TNM stage at diagnosis | RAI dose, mCi |
|-------------|-------------------------|-----|-----------|------------------------|--------------|
| 1           | 42                      | F   | PTC       | T1bN1M0                | 100          |
| 2           | 55                      | M   | HCC       | T2mN0M1                | 200          |
| 3           | 49                      | M   | PTC       | T1bmN1aM0              | 100          |
| 4           | 65                      | M   | PTC       | T2N1bM0                | 200          |
| 5           | 78                      | M   | HCC       | T4aN1bM0               | 200          |
| 6           | 50                      | F   | PTC       | T2mN1bMx               | 100          |
| 7           | 27                      | F   | PTC       | T3N1bM0                | 200          |
| 8           | 71                      | F   | PTC       | T1mN0M0                | 30           |

F, female; M, male; HCC, Hürthle cell carcinoma; PTC, papillary thyroid cancer; RAI, radioactive iodide.

Figure 2. Decreased plasma IGF2 concentrations 30 days after RAI treatment. (A) Blood plasma IGF2 concentrations in 8 patients with DTC pre-surgery compared with plasma IGF2 levels in 6 healthy volunteers. Data analyzed by Mann-Whitney U test. Blood plasma (B) IGF2 and (C) IGFBP2 concentrations in patients with DTC pre-surgery and 30 days after RAI treatment. Data analyzed by Wilcoxon matched-pairs signed rank test. DTC, differentiated thyroid cancer; RAI, radioactive iodide; IGF2, insulin-like growth factor 2; IGFBP2, insulin-like growth factor binding protein 2.
demonstrate the overexpression of IGF2 mRNA in undifferentiated thyroid cancer cell lines, poorly differentiated malignant thyrocytes, cancer thyrospheres and thyroid cancer specimens. This overexpression of IGF2 coincided with elevated expression of insulin receptor isoform A (IR-A) and insulin-like growth factor 1 receptor (IGF1R). They showed that this so-called IGF2/IR-A autocrine loop is associated with dedifferentiation and stem-like phenotypes, resembling RAI refractoriness (35-38). In line with these findings, we identified higher IGF2 expression in primary tumors from RAI refractory DTC patients via RNA sequencing. For the first time, our study also demonstrates a significant decrease of total IGF2 plasma concentration 30 days after primary therapy as compared to before surgery. Collectively these data suggest that IGF2 expression could have prognostic and therapeutic implications for several cancer types including DTC, which strengthens the importance of our findings and a potential role for IGF2 in acquiring RAI refractoriness.

The mechanism behind the increase in IGF2 expression in RAI refractory tumors demonstrated in this study is unknown. Previous studies have proposed several mechanisms to explain this increased expression causing therapy resistance. Wang et al showed that IGF2 produced by cancer-associated fibroblasts (CAFs) induced autophagy in cancer cells post-radiation thereby promoting cancer cell recovery (39). Also, drug resistance in non-small cell lung cancer cells was observed, inflicted by IGF2-AKT-Sox2-ABCB1 signaling in cancer cells co-cultured with CAFs (40). The survival of osteosarcoma cells, supported by IGF2, was dependent on enhanced autophagic flux and glutamine availability (27). In malignant rhabdoid tumors IGF2 has been shown to activate IGF1R and IR followed by activation of the PI3K-AKT and RAS-ERK pathways to promote proliferation and cell survival (41).

The present findings have potential clinical and therapeutic consequences. Several studies have been performed where IGF2 has been proposed as a predictive or prognostic molecular marker (42-44). A limitation to the use of IGF2 as a molecular marker for RAI refractoriness or for a more aggressive tumor behavior could be the presence of an autocrine IGF signaling loop as described by Vella and Malaguarnera (35-38). Measurement of serum IGF2 does not account for local IGF2 production in the tumors depending on an autocrine IGF2 signaling loop. Alternatively, measuring tumor IGF2 expression in the tissue available after thyroidectomy as we have shown in the study could be an potential option to be further explored (21).

Apart from serving as a molecular marker for RAI refractoriness, IGF2 could perhaps also be targeted therapeutically to overcome RAI refractoriness. Detecting increased circulating levels of IGF2 could be followed by targeting IGF2 or its receptors by using IGF monoclonal antibodies, IGF1R monoclonal antibodies or IGF1R/IR tyrosine kinase inhibitors. Multiple phase I/II trials in all types of cancers have been initiated using these therapeutic options, also in combination with chemotherapeutics and other drugs (21,45). Studies have shown that targeting the IGF1R combined with the IR pathway may increase therapy efficacy and prevents resistance to selective IGF1R antibodies or inhibitors (46,47). OSI-906, a dual inhibitor of the IGF1R and IR, has already shown antitumor activity in a phase I study and phase II studies in combination with other drugs are ongoing (48,49). In the case of DTC using such inhibitors could perhaps be beneficial in combination with RAI to overcome RAI refractoriness.

A few limitations of this study should be discussed. The retrospective design of our study and the criteria used for patient selection and RAI therapy indication possibly biased our results. Moreover, as mentioned earlier, the prevalence of distant metastasized DTC is <10%. Therefore, future studies will require larger sample sizes in addition to confirm our findings from both the retrospective cohort as well as the independent cohort. Furthermore, RNA-seq data from poorly differentiated TC cell lines such as FTC133, TPC-1 and BC-PAP showed in our hands a very low IGF2 expression in these cells. Since these cell lines are already RAI-refractory, overexpression or knockdown of IGF2 in these cell lines would not answer the questions regarding the potential role of IGF2 in acquiring RAI refractoriness.

In conclusion, important clinical, genetic and transcriptomic differences were identified between patients with RAI-sensitive DTC and RAI-refractory DTC. Interestingly, the tumor-promoting growth factor IGF2 showed a significantly higher expression in RAI refractory tumors. Plasma levels of IGF2 decreased following primary treatment in patients with DTC. These findings suggest that tumor-promoting growth factor IGF2 could be a potential factor in acquiring RAI refractoriness. Further studies in independent cohorts are needed to validate our findings and elaborate on the mechanism behind the elevated IGF2 expression and RAI refractoriness. Studies in independent cohorts using such inhibitors could perhaps be beneficial in combination with RAI to overcome RAI refractoriness.

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Availability of data and materials
The raw RNA datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus database under accession no. GSE112202 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112202).

Authors' contributions
TC, MHT, MJ and TSP performed the experiments and the data analysis. WEC and HM performed the gene-fusion experiments and analysis. KR recruited the patients, kindly provided the samples and clinical data for the independent cohort, and analysed the data of the independent cohort. ACHvEvG performed the pathological examinations of the tumour samples. TC, JWAS, RTNM and TPS designed the study. TC, JWAS, RTNM and TSP wrote the manuscript. TC and TSP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.
Ethics approval and consent to participate

Collection, storage and use of archived tissue and patient data were in compliance with the ‘Code of Proper Secondary Use of Human Tissue in the Netherlands’ (http://www.fmwv.nl and www.federa.org). The present study was approved by the Research Ethics Committee (CMO) of the Radboud University Medical Center under application no. 2015-1762 and followed the ethical guidelines of the CMO. The CMO waived the need for consent for the use of archived samples since the samples were analyzed anonymously. The independent cohort was approved by the CMO of the Radboud University Medical Center under application no. 2017-3628 (approval no. NLT03397238). Written informed consent was obtained from all participants and/or their legal guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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