Iodine avidity in papillary and poorly differentiated thyroid cancer is predicted by immunohistochemical and molecular work-up

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Word count: 3086
Short title: Iodine Avidity Prediction
Keywords: Thyroid cancer, iodine avidity, TERT, NIS, TPO

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Abstract

**Background** Successful radioiodine treatment of differentiated thyroid cancer requires iodine avidity: that is, the concentration and retention of iodine in cancer tissue. Several parameters have previously been linked with lower iodine avidity. However, comprehensive analysis on which factors best predict iodine avidity status, and the magnitude of their impact, is lacking.

**Methods** Quantitative measurements of iodine avidity in surgical specimens (primary tumour and lymph node metastases) of patients were compared to immunohistochemical expression of the TSH receptor, TPO, pendrin, NIS and mutational status of *BRAF* and the *TERT* promoter. Regression analysis was used to identify independent predictors of poor iodine avidity.

**Results** Mutations in *BRAF* and the *TERT* promoter were significantly associated with lower iodine avidity for lymph node metastases (18-fold and 10-fold, respectively). Membranous NIS localisation was found only in two cases, but was significantly associated with high iodine avidity. TPO expression was significantly correlated with iodine avidity (*r* = 0.44). The multivariable modelling showed that tumour tissue localisation (primary tumour or lymph node metastasis), histological subtype, TPO and NIS expression, and TERT promoter mutation were each independent predictors of iodine avidity that could explain 68% of the observed variation of iodine avidity.

**Conclusions** A model based on histological subtype, TPO and NIS expression, and TERT promoter mutation, all evaluated on initial surgical material, can predict iodine avidity in thyroid cancer tissue ahead of treatment. This could inform early adaptation with respect to expected treatment effect.
Introduction

Differentiated thyroid cancer is treated with surgery, and in cases of larger tumours or cervical lymph node metastases, additional hormone suppression and radioiodine therapy is given. Successful radioiodine therapy requires adequate uptake and trapping of iodine in the target tissue, which is conceptualised as iodine avidity. For most patients with low-risk disease, post-surgical treatment includes thyroid remnant ablation - a highly iodine avid target. However, in treatment of patients with known metastases, or at high risk of developing such metastases, the main target consists of distant or lymph node metastases of unknown avidity. Due to iodine avidity being unknown, it is currently the size of the primary tumour and presence of any initial lymph node metastases that mainly guides the choice of radioiodine treatment activity (1).

Several factors have been associated with lower iodine avidity of metastatic tissue, such as high patient age, large tumour size, histological type (follicular or papillary) and high \[^{18}\text{F}]\text{fluorodeoxyglucose uptake} (2–5). Furthermore, tumours exhibiting \textit{BRAF V600E} or \textit{TERT} promoter mutations are less likely to spawn iodine avid metastases and are associated with poorer patient outcomes (6–9). The co-occurrence of these two mutational events in papillary thyroid cancer (PTC) has been found to be especially indicative of aggressive tumour features (10).

The \textit{SLC5A5} gene encodes the sodium-iodide symporter (NIS) protein, which is central in the transport of iodine into thyroid cells, and its frequently decreased expression and function in thyroid cancer limits the effectiveness of radioiodine (11). Loss-of-function mutations in NIS are very rare in thyroid cancer, and the reduced iodine transport exhibited by most thyroid cancer cells appears to be related to silencing or post-translational changes prohibiting the essential plasma membrane localisation of NIS (12–14). Several other proteins have large impact on regulation and machinery of cellular iodine transport, such as thyroid peroxidase (TPO), thyroid stimulating hormone receptor (TSHR) and pendrin (\textit{SLC26A4}) (15).

However, all mentioned works linking various factors to iodine avidity have been based on post-therapeutic scintigraphic images. The classification of iodine avidity in previous research has been
based on regional and distant metastases, often as a binary parameter (uptake yes/no), which limits the ability to accurately quantify the uptake.

This study aimed to provide quantitative information on iodine avidity with higher precision and detail than previously published, using \textit{ex vivo} measurements of iodine concentrations in fresh tumour tissue from surgery. This enabled a unique evaluation of the iodine avidity in primary tumour tissue, resected lymph node metastases, and comparison of avidity to expression of iodine-transport related proteins (NIS, TPO, TSHR and pendrin), as well as mutations in the \textit{TERT} promoter and codon V600 of the \textit{BRAF} gene.

**Materials and methods**

**Patient selection**

Patients referred to the Karolinska University Hospital, Stockholm, Sweden, with cytologically confirmed PTC estimated as larger than 1 cm by ultrasound (the general threshold for radioiodine treatment at our institution), were informed and queried for study participation. All adult patients that could understand the study information were considered for participation. The study has been approved by the Swedish Ethical Review Authority (#2020-01222 and #2020-01541), and all subjects signed an informed consent form prior to inclusion. Exclusion criteria were pregnancy and severe renal impairment (eGFR <30 ml/min/1.7m$^2$). The patients were subsequently excluded if the primary tumour was too small and specimen collection at grossing therefore would risk compromising the histopathological diagnosis. Tissue specimens from 28 patients, collected between 2019 and 2021, were analysed. The data included primary tumour samples of PTC (21 patients), of differentiated high-grade thyroid cancer (DHGTC; 1 patient), of poorly differentiated thyroid cancer (PDTC; 3 patients), and lymph node metastases of PTC (11 patients), all removed at initial surgery. In eight of the eleven patients data on primary tumour and synchronous lymph node metastases was available;
in the remaining three patients, no data could be collected from the primary tumour due to small lesions or unclear localisation.

**Histological analysis**

Histopathological subtyping was performed in accordance with the upcoming 2022 World Health Organisation classification. Notably, it includes classification for the novel entity differentiated high-grade thyroid cancer: tumours with differentiated growth patterns and/or PTC associated nuclear changes, but with necrosis or an elevated number of mitoses (16).

**Sample and radioactivity handling**

The methodology of sample and radioactivity handling has been described in detail in a previous publication, studying other aspects of iodine avidity in a part of this cohort (17). Here follows a shorter summary, with more details in supplementary material S1. Two days prior to surgery, the patients received a low activity tracer injection of iodine-131 (5-10 MBq). The low activity and short time-period between injection and surgery ensured that absorbed doses to tumour tissue were far below (<0.1 Gy) what has previously been described to impact iodine uptake or NIS mRNA expression (18), see supplementary material S2 for details. After surgery, representative pieces of tumour and lymph node metastases were dissected by an experienced surgical pathologist or a specialised pathology laboratory assistant. In the case of multifocal primary tumour growth, the largest lesion was dissected. The radioactivity in tumour samples was quantified as normalised activity concentration (fraction of injected activity per gram of tissue: IA g⁻¹) by measurements in a gamma-counting scintillator detector. After radioactivity measurements were concluded, samples were fixed in formalin and embedded in paraffin (FFPE).

**Molecular pathology**
Digital droplet polymerase chain reaction (ddPCR) analysis was used to screen for TERT promoter mutations (both C228T and C250T), as ddPCR has shown superior sensitivity compared to Sanger sequencing (19). Positive mutation calling was noted if the fractional abundance exceeded 3%. The mutational status of the BRAF gene (exon 15) was analysed by direct sequencing according to standard procedures. BRAF mutation-positive cases were also interrogated for point mutations in the RAS gene family by examining codons 12, 13 and 61 for H-N-KRAS in order to exclude any competing driver mutations. More details of molecular pathology methods are described in supplementary material S1. Positive controls (Graves' disease), negative controls (without primary antibody) and plasma membrane specific stains performed for subsets of cases are shown in supplementary material S3.

**Immunohistochemical staining**

FFPE material from all thyroid samples were used for immunohistochemical analysis of NIS, TPO, TSHR, pendrin and, as a complement to PCR analysis, B-Raf V600E protein expression. The staining of each marker was assessed by an endocrine pathologist (C.C.J.). The level of immunoreactivity and sub-cellular localization was evaluated and scored on a scale of: negative (<10% of cells showing expression), weakly positive (25% of cells showing expression), moderately positive (50% of cells showing expression), strongly positive (75% of cells showing expression), and totally positive (100% of cells showing expression). More details of immunohistochemical staining methods can be found in supplementary material S1.

**Statistical analysis**

All analyses were performed using R (version 4.2.2, R-project.org). Data points on iodine avidity were log-transformed before statistical testing since their distribution was found to be approximately log-normal. For patients where multiple samples were taken from either primary tumour or lymph node metastases, the geometric mean of samples was calculated and used in any further analysis; only
the resulting mean value for either primary tumour or lymph node metastases were used in the analysis and shown in the Results section. For tests between dichotomous groups, Welch's t-test was used, since sample variance was not evidently equal between groups. Associations between continuous variables were estimated using Pearson's product-moment correlation. Multivariable regression was performed using a linear regression model (\textit{lm} function in R). The model was optimised with respect to maximising adjusted $R^2$ while keeping bias and number of variables as low as possible. The model parameters were chosen after an initial stepwise algorithm including all variables was reduced to decrease collinearity and number of variables, with the intention to limit any overfitting. Normality of residuals was evaluated with quantile-quantile plots. Multicollinearity in the model was evaluated using variance inflation factors (\textit{VIF} function in R). Skedasticity was evaluated using the Goldfeld-Quandt test (\textit{gqtest} function in R). Power calculations were performed with limited data on the variance in iodine avidity for the different subgroups; a standard deviation of 2.5E-5 IA g$^{-1}$ and effect size of 5E-5 IA g$^{-1}$ was used. A sample size of 25 was predicted to detect a two-fold difference in iodine avidity with 90% power in the rarest mutation (10% occurrence). In the Results section, two-sided 95% confidence intervals (CI) are reported throughout.

**Results**

*Links between iodine avidity, immunohistochemical expression and mutations*

Patient characteristics, the frequency of mutations in \textit{BRAF} V600E and \textit{TERT} C228T promoter mutations, and expression of NIS, TPO, TSHR and pendrin are shown in Table 1. Examples of immunohistochemical expression results for each antibody is shown in Figure 1. Mutational analysis and NIS staining failed in one primary tumour sample, TSHR and pendrin staining was inconclusive in one primary tumour sample each, and TPO was inconclusive in one primary tumour and in one lymph node metastasis sample. These samples are therefore not reported in Table 1.
BRAF V600E mutations were detected in 75% of primary tumors and in 55% of lymph node metastases. A statistically significant 18-fold lower avidity was observed in BRAF mutated lymph node metastases (CI 3.9-87). The TERT promoter C228T mutation was found in 33% primary tumors, in 36% lymph node metastatic samples. TERT promoter mutations in lymph node metastases was significantly associated with lower iodine avidity, with a 10-fold lower avidity (CI 1.7-60). The results for iodine avidity in patients with combined BRAF and TERT promoter mutations show a significantly lower avidity for lymph node metastases, with a 19-fold lower avidity for combined mutations (CI 3.4-110). All results for mutations are shown in Figure 2. No significant difference in iodine avidity was observed for either mutation in primary tumour samples. A single TERT promoter C250T mutation was found in one sample, that also exhibited a C228T mutation. Mutational status in primary tumours and lymph node metastases agreed in five out of eight, BRAF mutations were discordant in the remaining samples. No mutations in RAS genes were observed in the study population.

TPO expression had considerable variance in the cohort and correlated with both iodine avidity (r=0.44, CI 0.12-0.68), shown in Figure 3, and cytoplasmic NIS expression (r=0.42, CI 0.10-0.67). TPO expression was the only thyroid-related marker significantly associated with BRAF V600E (36 percentage points lower TPO expression, CI 13-58) and TERT promoter mutations (19 percentage points lower TPO expression, CI 3-35).

NIS expression was found in some primary tumors and the majority of metastatic lesions, as shown in Figure 4. The signal was predominantly cytosolic, with only two primary tumour samples demonstrating a clear-cut membranous signal. The samples with membranous NIS signal were BRAF and TERT promoter wildtype. The two samples with membranous NIS expression had a statistically significant 40-fold higher iodine avidity than those without membranous NIS expression (CI 9.1-180). No significant correlation was found between cytosolic NIS expression and iodine avidity (r=−0.23, CI −0.52 - 0.11). NIS expression was not found to be significantly different in patients with BRAF V600E or TERT promoter mutations.
TSHR expression was present to some degree in all samples, and pendrin was almost universally expressed (the only exceptions were two samples of PDTC). TSHR expression was moderately correlated with iodine avidity ($r=0.34$, CI 0.01-0.61) while pendrin showed no correlation with iodine avidity ($r=0.10$, CI $-0.24$ - 0.42).

**Multivariate regression**

The multivariate regression showed that a model using expression of TPO and NIS, mutation status of the *TERT* promoter, high-risk histology (tall cell PTC, PDTC and DHGTC) and tumour tissue localisation (primary tumour/lymph node) performed very well in predicting iodine avidity. The parameters of the model are shown in Table 2. The model had an adjusted $R^2$ value of 0.68 ($p<0.01$), implying that as much as two-thirds of the variation in iodine avidity between patients may be predicted from surgical material. The model had low collinearity (all variance inflation factors below 1.6), residuals were normally distributed and no heteroskedasticity was found (Goldfeld-Quandt test $p=0.72$). Removing most variables from the model caused a significant drop in adjusted $R^2$, but the model was found to perform similarly well (adjusted $R^2$ of 0.66) if *TERT* promoter mutation was replaced with Tg expression.

**Discussion**

This work includes, to the authors' knowledge, the first published data on the link between quantitative radioiodine avidity in surgical specimens, mutations in *BRAF* and the *TERT* promoter, and the expression of NIS, TPO, TSHR and pendrin. The unique study design enabled detailed quantification and modelling of iodine concentrations in the same tumour tissue that underwent histopathological examination.

The current work showed that several independent parameters coincide to explain why iodine avidity is found to be much lower in many thyroid cancers. Of major impact was the expression of TPO and
NIS, which can be expected because of their central part in concentrating and storing iodine in thyroid cells. Thyroid cancer cells are known to have variable and generally lower expression of TPO, which may have prognostic implications (20,21). The current work indeed found variable expression of TPO but our results also suggest that TPO can serve as a marker for the degree of iodine avidity in tumour tissue. NIS expression has been studied in relation to iodine avidity in metastatic lesions previously, finding links with iodine uptake on post-therapeutic whole-body scintigraphy (22–25). NIS mRNA levels have been linked to both TSHR mRNA and tumour marker Thyroglobulin mRNA levels (26,27).

Similarly to results from Tavares et al., the current work found NIS located at the basolateral membrane only in tumors of BRAF, TERT promoter and RAS wildtype (28). Since many samples in our cohort had concentrated iodine far above blood concentration, some level of NIS must have been present in the tumour cell membranes to mediate the transport, despite us being unable to detect it on immunohistochemistry with our methodology. Use of methods such as immunohistoflourescence or proximity ligation assays with higher sensitivity might enable further analysis of the presence of membranous NIS. Another possible explanation could be an unknown anion transport mechanism that may contribute to the observed iodine accumulation in cells with no observable membranous NIS. To exclude poor antigenicity at the plasma membrane level, subsets of NIS negative tumors were stained for E-cadherin and beta-catenin, all displaying strong and diffuse membranous staining - thereby suggesting that poor fixation is not a factor when determining NIS expression. Moreover, the risk of "stunning" was considered when choosing the activity of radioiodine prior to surgery. The used activities were well below what has previously been shown to not affect NIS mRNA levels (Supplementary material S2) (18).

The TSH receptor is known to vary in expression in thyroid cancer tissue, but to scarcely be absent, even in dedifferentiated tumours (29). TSHR expression was only found to be moderately correlated with reduced iodine avidity in this work and was not independent from other better-performing
parameters in the prediction of iodine avidity. Pendrin was substantially expressed in most samples in our cohort, regardless of iodine avidity, which is in line with previous research (30).

Lymph node metastases with \textit{BRAF} and \textit{TERT} promoter mutations were found to exhibit lower iodine avidity, with differences higher than previously reported. Previous studies have shown that the \textit{BRAF} V600E mutation, and the subsequent activation of the MAPK and PI3K/Akt/mTOR pathways, is linked to lower iodine avidity in metastases (31–34). Similarly, \textit{TERT} promoter mutations are associated with lower radioiodine uptake and worse patient outcome (35). Furthermore, the combination of \textit{BRAF} and \textit{TERT} promoter mutations may have a synergistic negative effect, with lower avidity and survival rates (6,8,9,36,37). The synergistic effect of both mutations was not observed in the current work. The results in the current work did show a similar trend as reported by Yang \textit{et al.} and Meng \textit{et al.} that calculated quantitative measures of tumour-to-background radioiodine uptake based on post-therapeutic scintigraphy (8,9). Their two studies found a 9-fold and 5-fold lower uptake in tumours harbouring \textit{TERT} promoter mutations. Yang \textit{et al.} also studied \textit{BRAF} mutations and found a 4-fold lower uptake in \textit{BRAF} mutated tumours. The differences in the current work were higher throughout, at 18-, 10- and 19-fold lower iodine avidity in lymph node metastases for mutations in \textit{BRAF}, \textit{TERT} promoter and their combination. This difference may be explained by larger dynamic range or lack of background signal in the current method or by chance, since the previous reported values reported by are encompassed by the reported confidence intervals.

The multivariable modelling shows that a substantial amount, perhaps up to two-thirds, of the variation in iodine avidity between patients can be accounted for ahead of initial radioiodine treatment. This has the potential to improve on current clinical management, where standard amounts (1.1, 3.7 and 7.4 GBq) of radioiodine is given, mainly based on pTNM staging. While TNM is a classification intended to stratify according to aggregated risk, the knowledge of iodine avidity enables
individualising treatment to the characteristics, and expected treatment benefit, of the individual patient.

One difference in the current work from the therapeutic setting is that no TSH stimulation was used. The patients were in a euthyroid state at the time of iodine-131 injection, which corresponds to lower TSH than in publications that studied post-therapeutic scintigraphies. Under strongly elevated serum TSH levels, such as after TSH stimulation in preparation for radioiodine treatment, iodine concentrations would probably have been higher, as TSH is known to mediate the transfer of NIS to the plasma membrane (38,39). It is worth noting that previous research has used therapy-related imaging after TSH stimulation and compared it to samples acquired under euthyroid conditions. Since in this study, all material was collected under a euthyroid state, the results add coherence to the analysis by performing specimen collection and avidity assessment simultaneously.

One strength of the current study is the use of tumoural iodine concentration as a measure of iodine avidity, which enabled both precise quantification and the molecular analyses to be performed on the same tissue. A limitation of the study is the relatively small number of participants, which may have hindered the detection of parameters with modest, but perhaps important, effect on iodine avidity. Further studies with larger patient series to confirm the predictive value of these parameters are therefore warranted.

In conclusion, we have shown that an extended immunohistochemical and molecular work-up can identify tumours with reduced iodine avidity in metastatic thyroid cancer using an ex vivo experimental design. The loss of avidity seems to be linked to loss of TPO expression, alterations in NIS expression, the gain of high-risk histology and TERT promoter mutations. These results could enable adaptation to expected radioiodine treatment effect and inform choice of radioiodine activity.
Declaration of interest

No potential conflicts of interest relevant to this article exist.

Author contributions

JNN: Conceptualisation, data collection, analysis, methodology, software, visualisation, writing;
JS: Conceptualisation, methodology, writing;
VC: Data collection, methodology, writing;
KJ: Data collection, methodology, writing;
RS: Data collection, methodology, writing;
CH: Conceptualisation, writing;
CIL: Conceptualisation, methodology, writing;
CCJ: Conceptualisation, data collection, analysis, methodology, visualisation, writing.

Funding

The study was financially supported by grants from the Swedish Cancer Society and Medical Diagnostics Karolinska.

Acknowledgements

The authors would like to thank Dr Daniel Thor for discussions and help with the statistical methodology.
References


Figure 1: Examples of immunohistochemistry for the studied iodine-transport related markers with hematoxylin (blue) as counterstain in all images. NIS expression (brown) in a sample of lymph node metastases is shown in (a). The sample was scored as 50% of cells expressing NIS. Expression of TPO (brown), scored as 50%, in a lymph node metastasis is shown in (b). TSHR expression (brown) was scored as 100% in a primary tumour sample in (c). Finally, pendrin (brown) was expressed in 75% of cells in a primary tumour sample shown in (d). The stroma present in the samples themselves served as negative controls for immunoreactivity. NIS - sodium iodide symporter; TPO - thyroid peroxidase; TSHR - thyroid stimulating hormone receptor.

Figure 2: Iodine avidity (injected activity per gram tissue) in relation to *BRAF* V600E (a) and *TERT* promoter C228T (b) mutations. Co-occurrence of mutations is shown in (c). Significant differences in avidity were found between wildtype and mutated samples of lymph node metastases (orange markers). + : positive, - : negative.

Figure 3: Iodine avidity (injected activity per gram tissue) in relation to proportion of tumour cells expressing TPO in primary tumour and lymph node metastasis samples. A significant correlation between iodine avidity and TPO expression of $r=0.44$ (CI 0.12-0.68) was observed. The log-linear fit is displayed along with confidence intervals (shaded area). TPO - thyroid peroxidase

Figure 4: Relation between proportion of cells in primary tumours and lymph node metastases with NIS expression as assessed by immunohistochemistry, and iodine avidity (injected activity per gram tissue). Separate data points are shown for cytoplasmic and membranous localisation of NIS staining. No significant correlation was found for cytoplasmic NIS expression and iodine avidity. The log-linear fit is displayed for cytoplasmic staining, along with confidence intervals of the fit (shaded area).
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41x41mm (1000 x 1000 DPI)
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Figure 4: Relation between proportion of cells in primary tumours and lymph node metastases with NIS expression as assessed by immunohistochemistry, and iodine avidity (injected activity per gram tissue). Separate data points are shown for cytoplasmic and membranous localisation of NIS staining. No significant correlation was found for cytoplasmic NIS expression and iodine avidity. The log-linear fit is displayed for cytoplasmic staining, along with confidence intervals of the fit (shaded area).
Table 1. Patient characteristics, histological subtypes, and frequencies of mutations and immunohistochemical expression. Immunohistochemical results are presented as number of patients with more than half of cells (≥50%) expressing the respective protein, except for membranous NIS. TPO - thyroid peroxidase; TSHR - thyroid stimulating hormone receptor.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total (n=28)</th>
</tr>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td>53 years (19, 81)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>11/17 (39/61%)</td>
</tr>
<tr>
<td><strong>Histological subtype</strong></td>
<td></td>
</tr>
<tr>
<td>Classic PTC</td>
<td>9 (32% of patients)</td>
</tr>
<tr>
<td>Diffuse sclerosing PTC</td>
<td>4 (14% of patients)</td>
</tr>
<tr>
<td>Warthin-like PTC</td>
<td>2 (7% of patients)</td>
</tr>
<tr>
<td>Follicular variant PTC</td>
<td>1 (4% of patients)</td>
</tr>
<tr>
<td>Oxyphilic PTC</td>
<td>1 (4% of patients)</td>
</tr>
<tr>
<td>Tall cell PTC</td>
<td>7 (25% of patients)</td>
</tr>
<tr>
<td>Differentiated high-grade thyroid cancer (DHGTC)</td>
<td>1 (4% of patients)</td>
</tr>
<tr>
<td>Poorly differentiated thyroid cancer (PDTC)</td>
<td>3 (11% of patients)</td>
</tr>
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**Frequency of mutations**

<table>
<thead>
<tr>
<th></th>
<th>BRAF V600E mutation</th>
<th>TERT promoter mutation (C228T)</th>
<th>BRAF and TERT mutation</th>
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</thead>
<tbody>
<tr>
<td>Primary tumours</td>
<td>75% (18/24 patients)</td>
<td>33% (8/24 patients)</td>
<td>33% (8/24 patients)</td>
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<tr>
<td>Lymph node metastases</td>
<td>55% (6/11 patients)</td>
<td>36% (4/11 patients)</td>
<td>36% (4/11 patients)</td>
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</table>

**Frequency of immunohistochemical expression**

<table>
<thead>
<tr>
<th></th>
<th>Cytosolic NIS expression (≥50% of cells)</th>
<th>Membranous NIS expression (any % of cells)</th>
<th>TPO expression (≥50% of cells)</th>
<th>TSHR expression (≥50% of cells)</th>
<th>Pendrin expression (≥50% of cells)</th>
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</thead>
<tbody>
<tr>
<td>Primary tumours</td>
<td>42% (10/24 patients)</td>
<td>8% (2/24 patients)</td>
<td>42% (10/24 patients)</td>
<td>88% (21/24 patients)</td>
<td>92% (22/24 patients)</td>
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<tr>
<td>Lymph node metastases</td>
<td>64% (7/11 patients)</td>
<td>0% (0/11 patients)</td>
<td>50% (5/10 patients)</td>
<td>64% (7/11 patients)</td>
<td>100% (11/11 patients)</td>
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</tbody>
</table>
Table 2. Results of the linear multivariable regression for iodine avidity. The model had an adjusted R2 of 0.68 and a p-value of <0.01. The model predicts that iodine avidity can be expected to be 40% higher for every 10% of cells that expressed TPO. Similarly, iodine avidity is modelled to be almost four-fold lower if the tissue specimen was of high-risk histological type. PTC - papillary thyroid cancer; PDTC - poorly differentiated thyroid cancer; DHGTC - differentiated high-grade thyroid cancer; TPO - thyroid peroxidase; NIS - sodium iodide symporter.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate (fold-difference)</th>
<th>95% Confidence Interval</th>
<th>p-value</th>
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<td>Tissue localisation (primary tumour)</td>
<td>8.6</td>
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<td>High-risk histology (tall cell subtype PTC, PDTC, DHGTC)</td>
<td>0.27</td>
<td>0.09 - 0.87</td>
<td>0.03</td>
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<td>TPO expression [per 10% of cells]</td>
<td>1.4</td>
<td>1.2 - 1.8</td>
<td>&lt;0.01</td>
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<td>Cytoplasmic NIS expression [per 10% of cells]</td>
<td>0.71</td>
<td>0.59 - 0.85</td>
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<tr>
<td>Membranous NIS expression [per 10% of cells]</td>
<td>1.4</td>
<td>1.0 - 2.1</td>
<td>0.08</td>
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<tr>
<td>TERT promoter mutation (C228T)</td>
<td>0.36</td>
<td>0.12 - 1.1</td>
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Supplementary material 1 - Material and methods details

Sample and radioactivity handling

Patients were asked to adhere to an iodine-restricted diet for one week before the injection, with the aim of limiting variance in uptake due to dietary iodine load. No thyroid stimulating hormone (TSH) stimulation was given to patients prior to the radioactive iodine, as patients receiving potent exogeneous TSH stimulation before surgery could risk having an induced thyrotoxicosis and subsequent higher risk of associated surgical complications. While the study methodology in general attempted to mimic the therapeutic setting where possible, the risks of TSH stimulation was not clearly outweighed by the potential benefits, as having the healthy thyroid present would still limit any direct comparison to the therapeutic setting.

After surgery, representative pieces of tumour and lymph node metastases were dissected by an experienced surgical pathologist or a specialised pathology laboratory assistant. In the case of multifocal primary tumour growth, the largest lesion was dissected. When feasible, multiple samples were taken from both the primary tumour and initial lymph node metastases to calculate averages of iodine concentrations, to limit the impact of random sampling in heterogeneous tumours. The iodine concentrations in representative tissue samples were measured using a calibrated NaI(Tl)-scintillation well chamber. The radioactivity in tumour samples was quantified as normalised activity concentration (fraction of injected activity per gram of tissue: \( \text{IA g}^{-1} \)).

Any non-tumoural components in the measured sample were corrected for by weight subtraction. The concentration was also corrected for competing uptake in normal thyroid tissue, as some patients had dominant accumulation of iodine in normal tissue. Therefore, similarly to biodistribution studies in small animals where activity can extravasate in significant amounts, the measured uptake was divided with the fraction of available iodine (excluding that in normal tissue) to improve comparison between patients.

Normal thyroid tissue taken at grossing served as an internal control for each patient, where the thyroid uptake could confirm the reliability of the measurements. The median normal thyroid tissue uptake was 38% after 2 days (IQR 25-52%), corresponding roughly to what could be expected in a population adhering to a low-iodine diet. The results of these control measurements are shown below in Figure 1.

Figure 1: Histogram of thyroid uptake measured in healthy gland at surgery. Whole-gland uptake was estimated by multiplying %IA/g (determined in representative samples) with whole-gland mass estimated at pathology grossing (total mass minus estimated primary tumour mass).
Molecular analyses

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections. Tissue blocks were cut and 3-6 sections 10 µm thick were used (number of sections depending on the tumor size). DNA was extracted with the FFPE DNA extraction kit using Maxwell RSC DNA FFPE KIT (AS1450) (Promega, Madison, Wisconsin, USA). Sections were then incubated in 180 ul of incubation buffer and 20 ul Protease K for two hours. After that, 400 ul of lysis buffer was added, and DNA was extracted using the Maxwell 16 robot (AS2000) (Promega, Madison, Wisconsin, USA). The quantity and quality of the DNA was measured by using Nanodrop (ThermoFisher Scientific, Waltam, Massachusetts, USA).

TERT promoter mutational analysis of DNA from FFPE samples was performed using ddPCR (QX200 Droplet Digital PCR system; Bio-Rad Laboratories, Hercules, CA, USA) together with validated ddPCR mutation assays for detection of mutations corresponding to upstream positions -124 (C228T) and -146 (C250T) of the TERT promoter region. The DNA input was 25 ng per reaction. Mutation-positive and mutation-negative references were male genomic DNA (Horizon Discovery, Cambridge, UK).

All samples were screened for BRAF V600E through PCR analysis on the Cobas z480 analyzer for automated amplification and detection using the Cobas 4800 BRAF V600 Mutation Test (Roche Diagnostics GmbH, Germany). All positive mutational findings were verified through additional real-time PCR screening for presence of hot spot mutations in KRAS (codons 12 and 13), NRAS (codon 61), and HRAS (codons 12, 13, and 61) with the EntroGen Thyroid Cancer Mutation Analysis Panel Kit (EntroGen, Woodland Hills, CA) using the protocol provided by the manufacturer.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissues from all thyroid samples were sectioned in 4 µm thick sections followed by deparaffinization in xylene, and rehydration in ethanol. All sections were subjected to antigen retrieval in tris-EDTA buffer, pH 8.0 (Sigma, E-1161) at 95 °C for 15 minutes. Slides were incubated at room temperature with hydrogen peroxide, followed by 15 minutes blocking step using Background Sniper (BS966; Biocare Medical).

Primary antibodies anti-TSH-R (1:2000; ab218108, Abcam), anti-TPO (1:4000; ab133322; Abcam), anti-SLC26A4 (1:2000; PA542060; Life Technologies), and anti-NIS (1:1000; ab242007; Abcam) were diluted in Renoir Red diluent (PD904; Biocare Medical) and incubated at 4°C overnight. MACH-1 Universal HRP-Polymer Detection kit (M1U539; Biocare Medical) was used for the detection step according to the manufacturing protocol. Counterstaining with hematoxylin as well as dehydration in ethanol and xylene was performed. De-identified cases of Graves’ disease patients were used as positive controls for each marker.

The mutation-specific V600E BRAF staining procedure was performed in a clinically accredited pathology laboratory setting using an anti-BRAF V600E (VE1) mouse monoclonal primary antibody and standardized methodology via a Ventana automated staining system (Roche, Basel, Switzerland). The staining was performed and scored in a clinical routine setting and was included in the analysis for validation purposes.

The plasma membrane proteins E-cadherin and beta-catenin were included to demonstrate intact antigenicity for membrane markers in subsets of NIS negative cases. Both antibodies are routinely used in our daily pathology practice at the Karolinska University Hospital, and were thus stained
using automated Ventana methodology. E-cadherin (clone 36) is a mouse monoclonal antibody used with a ready-to-use dilution with antigen retrieval using CC1 for 32 mins (Roche, Basel, Switzerland) and incubation time of 4 mins. Beta-catenin (clone 14) is a mouse monoclonal antibody used with a ready-to-use dilution with antigen retrieval using CC1 for 64 mins (Roche, Basel, Switzerland) and incubation time of 52 mins. External controls consisted on various de-identified tissues used in clinical routine (data not shown).
Supplementary material 2 - Absorbed dose to tumour tissue

Assumptions

Injection 10 MBq I-131 (max activity given)
Uptake 0.2%/g in tumour tissue (highest measured tumour uptake in the cohort)
Nothing leaves the tissue during the 48 h until surgery (conservative)
All beta energy stays in tumour tissue (slightly conservative)
192 keV deposited per decay

Results in:
0.1 Gy to tumour tissue
Lundh et al. (doi:10.2967/jnumed.108.061150) showed that at 0.5 Gy given with I-131 to normal thyroid cells in culture, no significant reduction in NIS mRNA expression could be observed at 1 d. An effect was seen at 5 d, however. Considering that 0.1 Gy was our "worst case", no stunning was expected to interfere with either the uptake (which in any case had already happened), nor NIS expression at surgery.

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<tr>
<th>Radionuclide</th>
<th>NIS mRNA downregulation (% of control)</th>
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<tbody>
<tr>
<td>¹³¹I</td>
<td>1 d  NS</td>
</tr>
<tr>
<td>¹²³I</td>
<td>55  NS</td>
</tr>
<tr>
<td>⁹⁹ᵐTc</td>
<td>34  NS</td>
</tr>
<tr>
<td>⁴ⁱ¹At</td>
<td>26  61</td>
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TABLE 2. Changes of NIS mRNA Expression in Thyroid Cells Irradiated with ¹²³I, ¹³¹I, ⁹⁹ᵐTc, or ²¹¹At to Absorbed Dose of 0.5 Gy During 6 Hours, on Days 1 and 5 After Irradiation

qRT-PCR data are presented as percentage downregulation, compared with matched nonirradiated controls. NS indicates no statistically significant difference from controls, \( P < 0.001 \).

FIGURE 3. Changes of NIS mRNA expression in thyroid cells irradiated with ¹²³I, ¹³¹I, ⁹⁹ᵐTc, or ²¹¹At at same dose (0.5 Gy) and exposure times as shown in Figure 2 (representing data from parallel cultures in same experiments) on days 1 and 5 after irradiation. qRT-PCR data are presented as log2 expression levels compared with those of matched nonirradiated controls \( n = 3 \). *Statistically significant difference from controls, \( P < 0.001 \).
Supplementary material 3 - Immunohistochemistry controls

Figure 1: Immunohistochemistry in four samples of Graves' Disease. NIS (a), TPO (b), TSHR (c) and pendrin (d) all showed intense signal as expected.
Figure 2: Immunohistochemistry in three of the studied tumour samples, omitting the primary antibody to confirm that no unspecific binding occurred.
Figure 3: Immunohistochemistry with cellular plasma membrane markers E-cadherin and beta-catenin.