A novel microfluidic device capable of maintaining functional thyroid carcinoma specimens *ex vivo* provides a new drug screening platform.

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Abstract

***Background**:* Though the management of malignancies has improved vastly in recent years, many treatment options lack the desired efficacy and fail to adequately augment patient morbidity and mortality. It is increasingly clear that patient response to therapy is unique to each individual, necessitating personalised, or 'precision' medical care. This demand extends to thyroid cancer; ~10% patients fail to respond to radioiodine treatment due to loss of phenotypic differentiation, exposing the patient to unnecessary ionising radiation, as well as delaying treatment with alternative therapies.

***Methods**:* Human thyroid tissue (n=23, malignant and benign) was live-sliced (5mm diameter x 350-500μm thickness) then analysed or incorporated into a microfluidic culture device for 96hr (37°C). Successful maintenance of tissue was verified by histological (H&E), flow cytometric propidium iodide or trypan blue uptake, immunohistochemical (Ki67 detection/ BrdU incorporation) and functional analysis (thyroxine [T4] output) in addition to analysis of culture effluent for the cell death markers lactate dehydrogenase (LDH) and dead-cell protease (DCP). Apoptosis was investigated by Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Differentiation was assessed by evaluation of thyroid transcription factor (TTF1) and sodium iodide symporter (NIS) expression (western blotting).

***Results**:* Maintenance of gross tissue architecture was observed. Analysis of dissociated primary thyroid cells using flow cytometry both prior to and post culture demonstrated no significant change in the proportion of viable cells. LDH and DCP release from on-chip thyroid tissue indicated that after an initial raised level of release, signifying cellular damage, detectable levels dropped markedly. A significant increase in apoptosis ($p<0.01$) was observed after tissue was perfused with etoposide and JNK

inhibitor, but not in control tissue incubated for the same time period. No significant difference in Ki-67 positivity or TTF1/NIS expression was detected between fresh and post-culture thyroid tissue samples, moreover BrdU positive nuclei indicated on-chip cellular proliferation. Cultured thyroid explants were functionally viable as determined by production of T4 throughout the culture period.

Conclusions: The described microfluidic platform can maintain the viability of thyroid tissue slices *ex vivo* for a minimum of four days, providing a platform for the assessment of thyroid tissue radioiodine sensitivity/adjuvant therapies in real time.

Keywords: Thyroid gland, Microfluidics, Viability, De-differentiation, Radioiodine therapy.

Introduction

Thyroid carcinoma (TC) is the most common malignant disease of the human endocrine system, accounting for ~2.1% of all cancer cases worldwide, and disproportionately affecting women (1). The incidence of TC has increased by 139% since the early 1990's in the United Kingdom, with a particular increase in the diagnosis of papillary TC (PTC; Cancer Research UK [2018]). PTC, along with follicular-TC (FTC), form the differentiated TC group (DTC), which represent ~90% of all thyroid malignancies (2). Patients with DTC have a 10-year survival rate of 90%, underpinned by an efficacious treatment regimen, which includes thyroidectomy followed by remnant and metastatic ablation using radioactive iodine (^{131}I).

Treatment, using ^{131}I, capitalises on the ability of thyrocytes to absorb iodide (I^-) for thyroid hormone biosynthesis. I^- is transported across the basolateral membrane of thyrocytes by the sodium iodide symporter (NIS) using the sodium gradient generated by Na^+/K^+-ATPase (3). Due to the essential involvement of the NIS in thyroid hormone biosynthesis and thus human metabolism in general, expression of the NIS is tightly regulated. In thyroid cancer, thyroid peroxidase (TPO) activity, and thus organification of free iodide is reduced. Oxidised iodine possesses a significantly longer retention time than free iodide, leading to a considerably reduced effective half-life in the malignant compared with the benign thyroid (0.5-3 days *vs.* 3-7 days, respectively; [4]). Despite this, treatment of DTC using ^{131}I remains an effective means of eradicating remnant and metastatic malignant foci when a high dose can be delivered to the target tissue (5). However, in approximately 15% of DTC cases, basolateral expression of the NIS is lost, due to phenotypic de-differentiation of thyrocytes, rendering ^{131}I therapy ineffective (6). Within this cohort of patients, the use of ^{131}I therapy is negated, as thyrocytes cannot concentrate ^{131}I, leading to a

marked reduction in patient survival (only 10% alive at 10-years, mean survival 3-5 years; [7]). Treatment of advanced, ^{131}I refractory TC using traditional systemic therapies including doxorubicin has limited efficacy with a high toxicity rate (8). Alternative therapy using relatively new-to-market tyrosine-kinase inhibitors (TKI), particularly sorafenib and lenvatinib, has garnered significant interest and has shown objective response rates with significantly improved progression-free survival (9,10). Currently it is not possible to predict accurately a lack of response to ^{131}I prior to administration, thus patients have a delay before receiving effective adjuvant therapy (11). Although it is well accepted that iodide uptake is typically reduced in malignant thyroid tissue relative to surrounding tissue (12), the molecular underpinnings have not yet been fully elucidated. A previous study by Min *et al.* (6) investigated the relationship between NIS expression and ^{131}I uptake in recurrent TC using immunohistochemical (IHC) analysis. They found that although lesional NIS expression predicted ^{131}I uptake with 100% specificity, the sensitivity of this method was just 50% (14 of 28 patients with positive ^{131}I uptake had negative NIS staining. Similarly, Arturi *et al.* (13) found, in a smaller study using RT-PCR, that only 4 out of 8 (50%) primary DTC with distant metastases and negative ^{131}I uptake lacked NIS gene expression in the primary lesion; thus indicating that assessment of NIS expression is certainly not an infallible means of evaluating ^{131}I uptake. Furthermore, Saito *et al.* (14) unexpectedly detected increased NIS mRNA and protein levels in PTC tissue compared with normal thyroid tissue. This variability suggests that reduced iodide uptake is perhaps due to failure to target or retain NIS protein at the basolateral membrane (15), rather than abrogated NIS protein production.

The microfluidic system described herein uses precision cut tissue slices (PCTS) which maintain the multicellular 3D architecture of the thyroid tissue

essential for intercellular communication, which is not well recapitulated in standard *in vitro* systems. Two previous studies have employed static culture of thyroid tissue explants: Russo *et al.* (16) investigated apoptosis subsequent to ^{131}I treatment in 1mm^3 tissue fragments, whereas Nagy *et al.* (17) examined the effect of various hormones and cytokines on the proliferation of 2mm^3 thyroid fragments. The results from these studies demonstrated that thyroid tissue cultured *ex vivo* can respond to external stimuli, however clinical utility was not assessed.

The bespoke device described in this manuscript mimics the *in vivo* vasculature and lymphatics by allowing continual perfusion of explanted tissue, along with removal of waste products and allows precise control of drug delivery (18). Future treatment of *ex vivo* thyroid tissue maintained in this device with ^{131}I, holds potential for a more realistic estimation of ^{131}I treatment success by assaying not only uptake, but resultant cell death. The aim of this study was to characterise a reproducible, easy to use, microfluidic system to maintain precision sliced *ex vivo* thyroid tissue for up to 96hr.

Materials and methods

Fabrication of a microfluidic culture device

Two polyether ether ketone plastic plates (PEEK; Direct Plastics, Sheffield, UK), both 30mm x 14mm in size (figure 1-A), were milled centrally to produce threaded axial holes to which inlet and outlet ethylene tetrafluoroethylene tubing (ETFE; 0.8mm internal diameter; Kinesis, IDEX Health & Science, Cambridge, UK) was attached via coned adapters (LabSmith, Mengel Engineering, Denmark). In addition, four ¼ inch (6.35mm) holes were made in both PEEK plates to allow the insertion of nylon screws in order to secure the unit after sample addition. A central cylindrical recess (10 x 4mm) was drilled in each PEEK plate to house a porous sintered pyrex disc (figure 1-A; The Lab Warehouse, Grays, UK). Finally, a silicone gasket (30mm diameter; 1 mm sheet silicone) with a 6mm central hole to create a tissue well, was placed between the two PEEK plates.

Sample collection and preparation

Human thyroid tissue samples (n=23 malignant; n=14 benign) were collected at the point of surgical resection during thyroidectomy following written informed consent under ethical approval from North East-Newcastle and North Tyneside Research Ethics Committee (15/NE/0412) and Hull and East Yorkshire NHS Trust R&D (R1925). Benign thyroid tissue was collected alongside malignant tissue when deemed practicable by consultant surgeon JE (n=14). Details of obtained tissue specimens are summarised in Table 1. Tissue samples were transported to the laboratory in ice-cold complete medium, then processed, allowing culture initiation within 60 minutes of surgical excision. Tissue was immobilised on a piece of cork and

156 sliced at a thickness of either 350 or 500µm in ice-cold PBS using a vibratome (Leica
157 VT1200S, Milton Keynes, UK) with a blade speed of 0.1 mm s^{-1} and amplitude of
158 2.5mm. A skin biopsy punch (Stiefel, Middlesex, UK) was used to generate PCTS
159 5mm in diameter. Each PCTS was weighed before insertion into the device. Where
160 samples sizes differ across experimental time-points this was due to difficulties in
161 effluent collection, sample numbers collected are provided in figures.

162

163 **Table 1: Disease and patient characteristics of thyroid specimens used in this study**

No	Tumour stage	Patient age	Gender	Tissue received
1	T2NXMX	45	M	M
2	T4aN1bMX	46	F	M
3	T3aNXMX	49	M	M
4	BG	56	M	B
5	T2NXMX	50	F	M,B
6	T3N1bMX	28	F	M
7	T4N0M0	80	F	M
8	T1aNXMX	64	F	M
9	BG	62	F	B
10	T2N1BMX	33	F	M,B
11	T3N1aMX	52	F	M
12	BG	34	F	B
13	BG	51	M	B
14	T4N0M1	51	F	M
15	T1aN1bMX	43	F	M
16	T1bNXMX	49	M	M,B
17	T3aNXMX	19	M	M
18	T3bN1aMX	44	M	M,B
19	T1NXMX	69	F	M,B
20	T1aNXMX	19	M	M
21	T3AN1bMX	32	F	M,B
22	T3aN1bM0	21	F	M
23	T1aNXMX	48	F	M,B
24	T3N1bM0	30	F	M,B
25	T2NXMX	73	F	M,B
26	T3aNXMX	62	F	M
27	T4aN1bMX	73	M	M,B

164 **BG**: Benign goitre; **M**: Malignant tissue; **B:** Benign tissue.

PCTS culture set up

Dulbecco's modified eagles medium (DMEM; GE Healthcare, Yeovil, Somerset, UK) containing 10% (v/v) heat inactivated foetal bovine serum (FBS; Biosera, East Sussex, UK), penicillin/streptomycin (0.1U/ml and 0.1mg/ml respectively; GE healthcare), 0.4mM glutamine (GE healthcare) and 2.5µg/ml Amphotericin B (Life Technologies, Paisley, UK) was supplemented with thyrotropin [TSH; 2 mIU/L (normal serum 0.3–5 mIU/L)] and sodium iodide (0.1µg/ml). 'Complete medium' was then loaded into a 20ml syringe and connected to the 2-part adapter and ETFE tubing via a 0.22µm filter (Figure 1; Millipore, Watford, UK). The PCTS was loaded onto a 70µm nylon porous membrane placed on top of the sintered disc in the inlet PEEK plate, and the outlet PEEK plate was then secured in place using nylon screws. The syringe was connected to a Harvard PhD 2000 syringe pump (Harvard, Cambridge, UK), which provided a pressure driven perfusion rate of 2µl min^{-1}. The culture device was maintained at 37°C inside an incubator for 96hr. Media coming off the chip was collected in 1.5ml polypropylene tubes after 2hr culture, then in 15ml polypropylene tubes once per day thereafter. Where required, for proliferation analyses, the thymidine analogue Bromodeoxyuridine (BrdU; Sigma-Aldrich, Gillingham, UK) was added, at 10µM final concentration, to the complete medium for the final 24 hours of culture (19).

Tissue embedding and morphological analysis

Fresh and post-culture tissue samples were either frozen in Tissue-Tek OCT (optimum cutting temperature; Sakura, Berkshire, UK) medium, using liquid nitrogen-cooled 2-methylbutane (Sigma-Aldrich), or fixed in 4% paraformaldehyde

(PFA) for 24hr. PFA fixed tissues were then dehydrated through an ethanol gradient (70, 90, 95, 100%), incubated with two changes of molten paraffin wax, then embedded and allowed to cool. Frozen tissue sections were cut at a thickness of 8µm using a Leica CM1100 cryostat and fixed for 20 minutes in -20°C cooled methanol before standard staining using Haematoxylin and Eosin (H&E; [20]). PFA fixed, paraffin embedded (PFPE) tissues were sectioned using a Leica RM2135 microtome (5µm).

Measurement of lactate dehydrogenase release

Lactate dehydrogenase (LDH) is a cytosolic enzyme released from the cell after plasma membrane damage (21). As membrane damage is synonymous with cell death, its release allows monitoring of the loss of cell viability (Cytotoxicity Detection Kit Plus, Roche, UK). Culture effluent (n=14 malignant, n=10 benign patient samples) was collected daily and stored at 4°C until the end of the culture period (96hr). In some cases (n=7) tissue was removed from the culture system at the end of the 96hr incubation and exposed to a 10% (v/v) lysis buffer overnight to induce cell death and subsequent LDH release. The assay was conducted following the manufacturer's protocol and results expressed as an average of duplicate readings normalised per mg of thyroid tissue.

Measurement of protease release

The CytoTox-Glo™ Cytotoxicity Assay (Promega, Southampton, UK) allows measurement of cell death using a luminogenic peptide substrate (alanyl-alanylphenylalanyl-aminoluciferin; AAF-Glo™ Substrate) to measure the activity of proteases released from non-viable cells that have lost membrane integrity. Culture effluent samples (including positive controls exposed to 10% (v/v) lysis buffer; 100µl

per well) were added in duplicate to a 96-well white-walled microplate. Malignant tissue samples were prioritised for use in DCP analysis. Assay Reagent (50µl) was then added to all wells and mixed briefly by orbital shaking. The reaction was incubated at room temperature for 15 minutes, before luminescence was measured using a Victor multilabel plate reader according to the supplier's protocol (n=7; PerkinElmer, Coventry, UK).

Viability analysis of dissociated cells

Malignant thyroid tissue was dissociated into a single cell suspension (n=3) prior to and after maintenance on the device by initial mincing using scalpels followed by a 2hr incubation (37°C; 5% CO_2) with 0.024% (w/v) Collagenase IV (Sigma-Aldrich: Dorset, UK) and 0.02% (w/v) DNase I (Roche, Herefordshire, UK) in complete medium under constant rotation. The resultant suspension was passed through a 70µm filter (BD Biosciences, Oxford, UK) prior to centrifugation at 400 x *g* for 5 minutes, to pellet the cells. The cell pellet was resuspended in 1ml of complete medium before viability was quantified using trypan blue exclusion. The cells were rinsed in PBS (Oxoid, Thermo Scientific, Hampshire, UK)/ BSA (2.5g/L; Thermo Scientific, Loughborough, UK)/Azide (0.0624% [v/v]; Sigma-Aldrich) and dead cells were stained by the addition of propidium iodide (500µg ml^{-1}). Cells were acquired immediately using a FACS Calibur flow cytometer (BD Biosciences) alongside unstained cells as a reference and results were analysed using Cell Quest Pro software, version 6.0.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay

Frozen tissue sections were defrosted for 5 minutes prior to fixing in 4% PFA for 20 minutes at room temperature. PFPE tissue sections were dewaxed in xylene (20 minutes) then rehydrated in 100%, 90% and 70% ethanol (2min per wash). Sections were treated for 20 minutes at 37°C (PFPE tissue) or 2 minutes on ice (frozen tissue) with 0.1% proteinase K/ 1% TrisHCl in dH_20. Sections were washed twice with PBS for 5 minutes and apoptotic cells were labelled using an *In Situ* Cell Death Detection Kit (Roche, UK) according to the manufacturer's guidelines. Negative control sections were incubated with 50µl label solution only, whereas test sections were incubated with both the label and enzyme solution. Sections were counterstained using a DAPI-hardset mounting medium (Vector Laboratories, UK) before fluorescence was evaluated using an ImageXpress Micro 4 Imaging System (Molecular Devices, California, USA). Produced tiled images were stitched to create a single composite image before apoptotic nuclei were quantified using ImageJ (Fiji plugin (22))

Treatment of thyroid tissue with etoposide and SP600125

Thyroid explants were exposed to etoposide and SP600125 for the complete (96hr) culture period in order to examine the ability of the system to induce a response which was then quantifiable using TUNEL staining. Etoposide, a topoisomerase II inhibitor, and SP600125, a JNK inhibitor, were added to complete culture medium at a concentration of 20µM, then perfused through the culture system under the same conditions as control samples.

Immunohistochemical analyses

Frozen tissue sections (8µm) were fixed in pre-cooled (-20°C) acetone for 20 minutes. Frozen tissue sections to be stained with anti-BrdU primary antibody were transferred into 2M HCl for 30min to denature the DNA and allow access of the antibody to the incorporated BrdU before being neutralised in borate buffer (0.2M boric acid, 0.05M sodium tetraborate [pH 8.4]; 2 x 5 min washes). PFPE tissue sections were dewaxed and rehydrated as previously described. Endogenous peroxidases were blocked in 3% (v/v) H_2O_2 (Fisher Scientific, Loughborough, UK) in acetone for 15 minutes. Antigen retrieval was carried out on paraffin embedded tissue sections by microwave boiling within Antigen Unmasking Solution (citrate based; pH 6.0, Vector Laboratories, Peterborough, UK) for 20 minutes**.** Slides were loaded into Sequenza™ racks and sections were incubated with normal horse blocking serum (Vectastain Elite ABC kit, Vector Laboratories) for 20 minutes. The addition of avidin D solution and, after a TBS wash, biotin solution (Vector Laboratories) for 15 minutes blocked non-specific binding of the avidin/biotin system.

Sections were incubated with primary antibody or matched isotype control antibody overnight at 4°C [Ki67 clone MIB1, 1:100 (Dako, Agilent, Stockport, UK)]; [BrdU clone 3D4, 1:50 (BD Biosciences)]; [TTF1 clone EP1584Y, 1:250 (AbCam)]. Following a TBS wash sections were incubated with secondary antibody (Vectastain Elite ABC kit) for 30 minutes followed by ABC detection reagent for 30 minutes. Colour was developed using 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and sections counterstained with Harris Haematoxylin. Sections were dehydrated and cleared through graded alcohols and histoclear™ (Scientific laboratory supplies, Nottingham, UK) then mounted using histomount (Scientific laboratory supplies).

The percentage of DAB positive cells was assessed across five separate fields using the online applet Immunoratio (http://153.1.200.58:8080/immunoratio/; (23)).

Assessment of NIS protein expression by Western blotting

Both fresh and post-culture thyroid tissue samples were minced using scalpel blades in ice cold RIPA buffer (Sigma-Aldrich) containing 1% (v/v) protease inhibitors (Roche) and sonicated for 1 minute before being agitated using a MACSmix™ tube rotator (2hr, 4°C; Miltenyi Biotec, Surrey, UK). Samples were then centrifuged at 16,000 x *g* for 20 minutes at 4°C, before the supernatant was aliquoted and frozen (-80°C). Proteins were quantified using a Pierce™ BCA Protein Assay Kit (Thermo Scientific) then 10µg was resolved on a 4% (v/v) stacking, 12% (v/v) separating sodium dodecyl sulfate–polyacrylamide gel in a Mini-PROTEAN® system, before being transferred onto nitrocellulose membranes using a Trans-Blot Turbo™ (both Bio-Rad, Watford, UK) and blocked in 4% bovine serum albumin (BSA) in TBS-Tween overnight. NIS was detected using a rabbit anti-human polyclonal antibody (ab199410, Abcam, Cambridge, UK) diluted 1:500. The house keeping gene β-actin was detected using a mouse anti-human antibody (sc-516102, Santa-Cruz Biotechnology, Heidelberg, Germany) diluted 1:10,000. Blots were developed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and Radiomat LS film (Agfa Healthcare, Middlesex, UK). Densitometry of bands was analysed using a Gel Doc system (Bio-Rad).

Measurement of thyroxine release by ELISA

Thyroxine (T4) is the principal hormone released by the thyroid gland, thus, its release from on-chip thyroid tissue was investigated using an enzyme-linked immunosorbent assay (ELISA, Abcam), according to the manufacturer's instructions,

as a measure of *ex vivo* functionality. Briefly, effluent samples (n=7 malignant, n=7 benign), controls and standards (25µl) were loaded in duplicate into a pre-coated 96 well plate, followed by 100µl working concentration of conjugate reagent. The plate was incubated for 60 minutes at room temperature. Each well was washed 5 times with distilled water before TMB (3,3',5,5'-Tetramethylbenzidine; 100µl) reagent was added before a further 20-minute incubation. The reaction was stopped by the addition of 100µl stop solution (2N Sulphuric acid [H_2SO_4] VWR, Leicestershire, UK) to each well. Absorbance was measured at 450nm using a Multiskan plate reader (Thermo Scientific). T4 concentration in each sample was determined from the standard curve and normalised per mg of thyroid tissue.

Results

The morphology of both freshly resected and post-culture (96hr) *ex vivo* thyroid tissue sections was assessed by H&E staining and showed preservation of gross morphology, with retention of tissue cohesion (figure 2). The follicular structure, typical of *in vivo* thyroid tissue, was clearly displayed in both fresh and post-culture tissue samples. Inter-tumour variation in cellularity and follicle size is clear, however these differences are retained in tumour tissue throughout culture.

Culture effluent was sampled throughout the 96hr culture period and examined for the presence of two markers of cell death, LDH and DCP. An initial raised level of LDH release was observed within the first 2hr of culture setup (figure 3A), which reduced after 24hr and remained consistently low for the remaining 72hr, until tissue was exposed to 10% (v/v) lysis buffer, which led to an average increase in LDH release from 0.01 ±0.002 to 0.304 ±0.06 and 0.005 ±0.001 to 0.35 ±0.03 absorbance units mg^{-1} in malignant and benign thyroid tissue, respectively. No difference in LDH

release was found between the malignant and the benign tissue. DCP release by cells in the culture system followed a profile similar to that of LDH release (figure 3B), with the level being relatively high in the first 2hr of culture before decreasing after 24hr. DCP release reached a minimum after 72hr, with only 41.53 ± 28.33 RLU mg^{-1} detectable. Concordant with LDH release, purposeful lysis of cultured thyroid tissue subsequent to 96hr culture induced a >1000-fold increase in detectable DCP.

No significant difference in viability between fresh (27.3 ±7.3%) and post-96hr cultured (24.6 ±8.8%) malignant thyroid tissue samples was observed using PI staining to (Figure 4; n=3; p=0.19). These data were consistent with results using trypan blue exclusion to represent cell viability, whereby the mean proportion of viable dissociated cells was 38.33 ±7.3% in fresh thyroid tissue prior to culture and 34 ±9.5% in tissue after a 96hr culture period (figure 4; n=3; p=0.37). The proportion of apoptotic cells in both fresh and post-culture (+96hr) malignant thyroid tissues was assessed by TUNEL staining (n=9) and a small, non-significant, increase in apoptotic cells was observed in the post-culture tissue (5.59±0.99%) compared with the fresh tissue (2.77±0.89%; figure 4-C and D). Moreover, on-chip treatment of thyroid explants with etoposide (topoisomerase II inhibitor) and SP600125 (JNK inhibitor) caused a significant increase in cellular apoptosis after 96hr treatment, compared to untreated thyroid tissue (15.25% and 14.24% vs. 5.59%; p=0.0025 and p=0.0069 respectively; figure 4).

Cell proliferation within the thyroid tumour tissue was investigated by IHC. Two markers were used; Ki67, a nuclear protein expressed during all active phases of the cell cycle (non-senescent cells) and BrdU which is incorporated during s-phase of the cell cycle (24). This analysis demonstrated that proliferating cells were detectable in both freshly resected tissue and post-culture tissue. The mean percentage of Ki-67

positive nuclei was 1.45±0.33% and 1.32±0.34% in fresh and post-culture tissue (n=8), respectively; no significant difference in the quantity of proliferating cells between fresh and post-culture malignant thyroid tissue was observed (p=0.32). BrdU, which was perfused through the culture system for the final 24hr culture on the device, was clearly visible as incorporated into the cell nuclei of proliferating cells post-culture, illustrating that on-chip cell proliferation was occurring (figure 5). Subsequent to a 24hr perfusion with 10μM BrdU, 12.09±4.38% cells stained positive for BrdU incorporation (n=3). Thyroid transcription factor 1 (TTF1) is crucial for thyroidal organogenesis and differentiation which, along with two other factors, paired box gene 8 (PAX8) and TTF2 (FOXE1), governs the expression of thyroglobulin (TG), thyroperoxidase (TPO) and NIS (25). The expression of TTF1 was characterised in fresh (0h) and post-culture (+96hr) malignant thyroid tissue; the staining pattern of TTF1 was maintained throughout culture in the microfluidic device (figure 5).

As expression of the NIS is crucial for adequate response to treatment to I^{131}, it was important to ensure that 96hr culture of malignant thyroid explants within our MF device did not affect NIS protein levels. NIS expression was investigated in both freshly resected and post-culture malignant thyroid tissue and showed that there was no statistically significant difference between the density of NIS protein bands in fresh and cultured thyroid tissue, normalised to β-actin (figure 6; n=5), indicating that the expression of NIS was maintained throughout culture.

The functionality of cultured *ex vivo* thyroid tissue was investigated by its capability to synthesise and release the primary thyroid hormone, T4. Production of T4 occurs as a result of a cycle of biochemical reactions occurring exclusively within the thyroid. Culture effluent was analysed by ELISA for the presence of T4 (n=7

malignant tissue; n=7 benign tissue). Data indicate that T4 release by both malignant and benign thyroid explants follows a similar overall trend, with an initially raised level of T4 production over the first 24hr, stabilising after 48hr (figure 7). Interestingly, T4 release by benign thyroid explants was consistently higher at each timepoint in comparison to malignant tissue and average T4 output throughout the complete culture period was significantly higher in benign tissue (0.29±0.09 µg/dl mg^{-1}) than in malignant tissue (0.14±0.03 µg/dl mg^{-1}; figure 7; p=<0.05).

Discussion

The current study aimed to characterise the ability of a PCTS device to maintain viable thyroid tissue explants over a 96hr period with a view towards therapeutic interrogation. Human tissue explants from human head and neck tumours, intestine and mouse brain have been maintained using similar microfluidic culture systems (20,26,27) however this is, to the best of the authors' knowledge, the first time *ex vivo* human thyroid tissue has been cultured in a robust, easy-to-use, platform. Tissue sections taken from both fresh and PCTS-cultured thyroid tissue analysed by haematoxylin and eosin and IHC staining demonstrated that morphology was preserved in tissue which had been cultured in the device for 96hr, validated by a consultant histopathologist. Results observed agree with those shown in similar studies from the group, whereby head and neck squamous cell carcinoma and full thickness intestinal tissue also demonstrated a preservation of tissue morphology after 72hr on-chip culture (18, 20). The gross inter-patient morphology of tissue specimens was variable, and heterogeneity was visible within each tissue sample. Areas of

typical thyroidal histology exhibiting follicular architecture often neighboured regions of densely arranged cells or fibrous stroma. This inter- and intra- patient heterogeneity was expected and was also presented in a study by Radu *et al*. (28), who speculated that the morphological variation in thyroid carcinoma is due to manifestation of diverse tumour cell clones under the influence of the same etio-pathogenic factors. It will therefore be necessary to run multiple PCTS chips from the same patient when interrogating the tissue with different treatment regimens.

The release of both LDH and DCP as markers of cell membrane integrity was assayed throughout the culture period; loss of cellular plasma membrane integrity is synonymous with loss of cellular viability (29) and microfluidic culture offers a unique means of evaluating this in real time. As anticipated, the release of these two markers followed similar patterns, with a higher level at the beginning of the culture period, attributed to the damage caused to the tissue during preparation, which decreased during the remainder of the experiment. The relatively low LDH/ DCP release once the tissue had recovered from the initial cellular damage during set-up, which has also been experienced in previous studies (30,31), indicates cultured explants do not undergo further degradation due to factors such as sheer stress or lack of oxygen within the culture chamber. Deliberate lysis of cultured tissue resulted in an increase in both LDH and DCP, suggesting that a significant number of viable cells remain after 96hr in the PCTS device, which agrees with the morphological assessment. A similar pattern has been observed studies from other groups where stabilisation of LDH release was seen after four days in a four-organ-chip model (32) and after six days in Wagner *et al.'s* liver/skin co-culture (33). The discrepancy in time taken for tissue to recover from the initial damage could potentially be

attributable to either the difference in tissue type, the system used or the presence of malignancy in the current system.

Tissue dissociation using a combined mechanical and enzymatic method offered an alternative means of investigating the survival of individual cells constituting the gross thyroid explants, by monitoring the ability of propidium iodide and trypan blue to traverse the cellular membrane. Again, no statistically significant difference in mean cellular viability was observed between samples which had been dispersed prior to, or after PCTS-culture, further demonstrating that on-chip maintenance was not causing increased cell death. This was despite the fact that the overall viability of cells after dispersal from gross tumour material was unexpectedly low (<40%), presumably due to injury caused by the enzymatic (collagenase IV, DNase II) and mechanical (mincing of gross tissue using scalpel blades) process of dissociation. The high level of cell death observed in the current study is in contrast to Li *et al*. (34) who provided a mean cell viability of 65.66% (±4.96) using trypan blue staining following disaggregation of murine pancreatic fragments using collagenase IV. As malignant tumour tissue is marked by regions of necrosis owing to rapid oncogene-driven proliferation not supported by an adequate vascular bed (35), it is a possibility that presence of necrotic cells contributed to the lower overall viability in the current study. The viability of cells dispersed from human head and neck cancer specimens was assessed by flow cytometry in a separate study and produced viabilities ranging from 24-92% (36), partially aligning with the results seen here.

To further characterise on-chip tissue kinetics, the proportion of thyroid cells undergoing apoptosis was elucidated through the labelling of 3-OH termini of DNA strand breaks *in situ*, produced due to endonuclease mediated DNA fragmentation, characteristic of DNA degradation and late-stage apoptosis (37). The culture of

thyroid explants within the microfluidic device showed no change in the proportion of apoptotic cells. Similar studies have, however, reported comparable levels of apoptosis in untreated specimens' post-microfluidic culture. Cheah *et al.* (22), found an apoptotic fraction of <10% within untreated head and neck squamous cell carcinoma (HNSCC), whilst Wang *et al.* (38) also saw <10% apoptosis post-culture in brain organoids, compared to ~40% apoptosis in organoids subjected to static culture conditions. The increase in apoptosis observed in this study was not mirrored in other tests of viability such as PI/TB uptake and LDH/DCP leakage data as the latter assays rely on loss of membrane integrity, synonymous with necrotic cell death (38), to facilitate detection of leaked cellular components (LDH/DCP) or PI/TB labelled cells. It is feasible that, if cultured for an extended period of time, a decrease in cell viability would also be observed using PI/TB uptake and LDH/DCP leakage assays, due to the occurrence of cell death by alternative mechanisms such as secondary necrosis, characterised by progressive loss of plasma membrane integrity, through caspase-3-dependent cleavage of the GSDMD-like protein DFNA5 (38,39). Furthermore, on-chip treatment of thyroid explants with 20µM etoposide and SP600125 demonstrates the devices' utility as a drug screening system. Perfusion of thyroid explants with either agent for 96hr resulted in significantly increased levels of apoptosis as examined by TUNEL staining. Etoposide is a well-characterised inducer of apoptosis (40) whilst SP600125 is a multikinase inhibitor which has been shown to have both anti-proliferative and migratory effects in thyroid cell lines (41).

IHC analysis was used to investigate the ability of the cells within the cultured tissue to proliferate. The Ki67 antigen is present in all active stages of the cell cycle, except for $G_{0,}$ in which cells are in a quiescent state (42). Previous studies have attempted to use Ki67 labelling index to aid diagnosis in thyroid neoplasms, however

it has shown little value in this setting due to the low labelling index in most thyroid lesions (<1% in 57% PTC samples tested; [43]). In agreement, Ki67-positive brown stained cells were detectable at low levels (<2%) in tissue sections in the current study after 96hr within the PCTS device indicating the presence of a small number of proliferating cells. Furthermore, comparison of the proliferating cell population in both fresh and post-culture thyroid tissue revealed no significant difference.

BrdU staining was also employed, having the added advantage of demonstrating definitive on-chip division as BrdU is incorporated into *de novo* DNA during the S-phase of the cell cycle as a substitute for thymidine and is detectable by IHC (44). Cells with BrdU positive nuclei (12.09%) were evident in thyroid tissue samples which had been maintained for 96hr in the PCTS-culture device, the final 24hr of which included perfusion with 10µM BrdU. Identification of BrdU-positive cells throughout the tissue both demonstrates the ability of BrdU to perfuse successfully through the tissue and moreover the presence of proliferating cells in the S-phase of the cell cycle. A comparable study by Sart *et al.* (45) investigated BrdU uptake in H4-II-EC3 rat hepatoma cells cultivated in both a 2D and 3D spheroid setting. The authors saw a near 2-fold decrease in BrdU positivity in 3D (~16%) versus 2D (~30%), similar to results observed in this study.

In addition to the viability of the tissue, it was important to characterise the differentiation of thyroid explants, in order to examine whether culture within the microfluidic device could affect the expression of certain genes crucial for successful treatment with radioiodine. A previous study conducted by Cirello *et al.* (46) which aimed to investigate the effects of the multikinase inhibitor SP600125 on multicellular spheroids derived from primary thyroid tissue, examined expression of the transcription factor TTF1 as a marker of differentiation. The authors observed

505 maintenance of TTF1 expression in spheroids derived from primary thyroid tissue.
506 Encouragingly, the pattern of TTF1 staining in post on-chip culture (+96hr) thyroid
507 tissue was equivalent to that of fresh thyroid tissue, suggesting that culture for 96hr in
508 the PCTS device did not markedly affect differentiation.

509 The NIS is an integral plasma membrane glycoprotein that facilitates the
510 accumulation of I^- within the thyroid gland at a concentration up to 40 times higher
511 than plasma (47). Application of I^{131} for the targeted destruction of malignant
512 thyroidal tissue has become one of the most effective radiation regimens available,
513 causing fewer side effects than other cancer therapies (48). Loss of NIS protein
514 expression at the basolateral membrane of thyrocytes is a well-accepted precursor for
515 reduced effectiveness of I^{131} therapy, as demonstrated in poorly differentiated and
516 undifferentiated thyroid cancers (49,50). Therefore, it was necessary to assess the
517 expression of NIS in thyroid explants which had been subjected to 96hr within the
518 microfluidic device and elucidate whether its expression was altered. Western blot
519 data revealed that there was no significant change in terms of NIS protein levels
520 caused by culture within the PCTS device, indicating that the system is valid for the
521 future testing of I^{131} sensitivity.

522 T4 is exclusively synthesised by thyrocytes as a pro-hormone, which becomes
523 de-iodinated within target tissues to produce the more biologically potent T3 (51).
524 This natural phenomenon allowed the measure of T4 release from on-chip PCTS to be
525 investigated as a measure of tissue functionality. Loss of, or reduced functional
526 differentiation, occurs in all thyroid carcinomas leading to diminished expression of
527 proteins central to thyroid hormone production (52). In the current study, benign
528 tissue produced a significantly higher volume of T4 than malignant tissue throughout
529 the culture period, agreeing with the notion that malignant thyroid tissue has a

530 decreased ability to produce hormone (11). Detection of T4 within culture supernatant
531 strongly suggests that thyroid hormone was synthesised *de novo* by on-chip thyroid
532 tissue and that essential components such as I^- are reaching the thyrocytes. In the
533 clinical setting, the thyroid hormone precursor TG is used as a specific marker of
534 remnant tumour tissue after thyroid removal (53). As our intention was to demonstrate
535 thyroid tissue functionality, we decided to measure T4 levels as T4 synthesis relies on
536 the success of additional mechanistic steps such as cellular I^- uptake and TG
537 iodination.

538 **Conclusion**

539 Results reported in this work validate the use of a PCTS device for the
540 maintenance of *ex vivo* thyroid tissue which can be used for customising drug
541 treatment. It is proposed to use the system described to delineate sensitivity of patients
542 suffering from thyroid carcinoma to treatment with ^{131}I, enabling patient stratification
543 and reducing incidence of inappropriate treatment, reducing patient morbidity, whilst
544 saving NHS resources. This is the first study of its kind to demonstrate reliably the
545 maintenance of viable *ex vivo* thyroid tissue for up to 96hr, with pre- and post-culture
546 tissue showing high uniformity in terms of morphology, viability and functionality.

547 **Abbreviations**

548 TC: thyroid carcinoma; PTC: papillary thyroid carcinoma; FTC: follicular thyroid
549 carcinoma; DTC: differentiated thyroid carcinoma; I^{131}: radioactive iodine; NIS:
550 sodium-iodide symporter; TPO: thyroid peroxidase; TKI: tyrosine kinase inhibitor;
551 IHC: immunohistochemistry; RT-PCR: real time-polymerase chain reaction; mRNA:
552 messenger ribonucleic acid; BSA: bovine serum albumin; PCTS: precision cut tissue
553 slice; PEEK: polyether ether ketone; ETFE: ethylene tetrafluoroethylene; PBS:

phosphate buffered saline; BrdU: bromodeoxyuridine; OCT: optimal cutting temperature; LDH: lactate dehydrogenase; DCP: dead cell protease; T4: thyroxine; TMB: 3,3',5,5'-Tetramethylbenzidine; HCl: hydrochloric acid; DAB: 3, 3 – diaminobenzidine; TTF1: thyroid transcription factor-1; TG: thyroglobulin; DNA: deoxyribonucleic acid.

Declarations

Ethics approval and consent to participate

The project had received ethical approval from North East-Newcastle and North Tyneside Research Ethics Committee (15/NE/0412) and was approved by Hull and East Yorkshire NHS Trust R&D (R1925).

Consent for publication

Not applicable.

Availability of data and material

All data is provided within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AR, VG, and RC undertook the experimental work; AR, JE, VG and JG designed the project; GM and JE collected the thyroid specimens and relevant clinical data; LK

provided the histological analysis. JG is responsible for guaranteeing the data. All authors approved the final version of the manuscript.

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Figure Legends

Figure 1: **PCTS Culture device. A:** Photograph illustrating device assembly. Two polyether ether ketone (PEEK) plastic plates, each with a medial recess to house a sintered pyrex disk, affix to sandwich a silicone gasket with a central cavity in which the precision cut tissue slice (PCTS) is contained. A 70µM porous nylon membrane separates the tissue from the sintered disk (not shown). **B:** Photograph of an assembled PCTS culture device with external dimensions. **C:** Schematic of PCTS culture device. Component parts numerically labelled: 1, PEEK plastic plates; 2, glass sintered pyrex disk; 3, central silicone gasket; 4, tumour tissue sample.

Figure 2: **Haematoxylin and eosin stained 8µM frozen thyroid sections**. Representative images from two patients A and B. **Top**: fresh tissue frozen prior to culture. **Bottom**: tissue frozen subsequent to 96hr culture within the PCTS device. Images are shown at both 100x and 400x magnification.

Figure 3: **Measurement of lactate dehydrogenase (LDH) and dead-cell protease (DCP) release into culture effluent for the evaluation of cell membrane damage. A:** Lactate dehydrogenase release from both malignant and benign thyroid tissue (n=14 and n=10, respectively unless otherwise stated; relative absorbance of formazan reaction product) during on-chip culture of thyroid tissue normalised per mg starting tissue wet weight. **B**: Level of relative light units (RLU) produced as a result of cleavage of the luminogenic assay substrate by dead cell protease released per mg of on-chip thyroid tissue over the 96hr culture period (n=11). Lysis buffer (10% v/v) was added to homogenised tissue after 96hr culture (arrow) in order to purposefully rupture cell membranes and induce complete release of remaining LDH/ DCP.

Figure 4: **Cellular viability and apoptosis in freshly resected, control and treated thyroid tumour tissue**. Cellular ability to prevent both PI (**A**) and TB (**B**) transit across the plasma membrane was employed as a proxy of viability (n=3, mean+ SEM). No significant difference was observed between fresh and untreated control thyroid tissue. **C:** Terminal deoxynucleotidyl transferase dUTP nick end labelling in both fresh (+0h) and post-culture (+96hr) malignant thyroid tissue explants demonstrated no difference in the proportion of apoptotic cells in post-culture specimens (n=9; 2.78 *vs*. 5.59%, p=0.51 [paired t-test]*).* Treatment of cultured thyroid explants with etoposide and SP600125 resulted in a significant increase in apoptosis (15.25% and 14.24%, respectively) which were both significantly increased compared to untreated explants (n=5; p=<0.001). **D:** Representative images of both pre-MF and untreated post-MF thyroid explants illustrating apoptotic cells (FITC; green) counterstained with DAPI (blue).

Figure 5: **Percentage Ki-67 positivity in fresh (+0h) and post-culture (+96hr) thyroid tumour tissue**. Mean Ki-67 positivity was 1.52 ±0.52% and 1.3 ±0.34% in fresh and post-culture tissue, respectively; no significant difference was observed (n=8; p=0.32 [paired t-test]). **Inset**: Representative photomicrographs of Ki67, BrdU and TTF1 positive brown nuclei and counterstained with haematoxylin (x400). BrdU staining is only shown in cultured tissue due to the requirement of perfusion with the molecule in order to elicit its incorporation and subsequent detection; 12.09 ±4.38% cells stained positive for BrdU incorporation (n=3).

Figure 6: **Assessment of sodium iodide symporter (NIS) protein expression by Western blotting.** Band densitometry confirmed that NIS expression was not significantly altered by the microfluidic culture of thyroid tumour tissue explants (n=5). [+] and [–] represent protein extracted from tumour tissue with and without on-chip culture respectively. 1-5 indicates five separate patient samples and K1 is protein extracted from the thyroid cell line.

Figure 7: **Thyroxine (T4) release by on-chip thyroid tissue**. Concentration of T4 in effluent samples collected throughout the culture of both malignant (n=7) and benign (n=7) thyroid tissue explants on the PCTS device. Overall T4 release by benign thyroid tissue was significantly higher than that of malignant thyroid tissue (p=0.03; 2-way ANOVA). Results normalised mg^{-1} tissue.