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## Detection of Circulating Thyroid Tumor DNA in Patients with Thyroid Nodules

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Surgery

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## Abstract

This study was conducted to determine the feasibility of detecting *BRAF(V600E)* circulating tumor DNA (ctDNA) in the plasma of patients with thyroid nodules, with the goal of distinguishing between benign and malignant nodules. Plasma samples from patients undergoing thyroid surgery for thyroid nodules were obtained prospectively pre-operatively and one month post-operatively, and quantitative-PCR was used to determine the presence of *BRAF(V600E)* ctDNA. These were compared to formalin fixed paraffin embedded samples from the index nodule. Thirty-eight pairs of preoperative and postoperative plasma samples were collected and analyzed. 6/18 (33.3%) patients with classical PTC and 0/8 (0%) patients with nodular hyperplasia had detectable levels of *BRAF(V600E)* ctDNA pre-operatively, thus *BRAF(V600E)* ctDNA was able to distinguish between benign and malignant nodules ( $p < 0.05$ ). The levels of all samples with detectable *BRAF(V600E)* ctDNA pre-operatively declined post-operatively ( $p < 0.05$ ). *BRAF(V600E)* ctDNA can be detected in plasma. Post-operative drop of *BRAF(V600E)* ctDNA in all cases suggests its utility as a tumor marker.

## Keywords

Circulating tumor DNA, cell free DNA, ctDNA, thyroid cancer, thyroid nodule, *BRAF(V600E)*

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## 1. Introduction

There is an increasing incidence of thyroid cancer in developed countries, with approximately 75% of these cases occurring in women. Although approximately 50% of thyroid glands have thyroid nodules when examined at autopsy, during surgery or by ultrasound, approximately two-thirds of these are benign.<sup>1</sup> Up to 35% of thyroid glands removed at autopsy or surgically have clinically unimportant thyroid cancers, primarily papillary thyroid cancers (PTC) less than 1.0 cm.<sup>2,3</sup> It is the most common endocrine malignancy worldwide and tenth most common cancer in Canada.<sup>4</sup> In Canada, from 1986 to 2010, the age standardized incidence rate (ASIR) for thyroid cancer increased from 2.0 to 5.9 per 100,000 in males and from 5.2 to 20.6 per 100,000 in females (Figure 1).<sup>5</sup> There was a 6.3% per year increase in incidence of thyroid cancer in males from 2001 to 2010 and 4.4% per year increase in incidence of thyroid cancer in females from 2005 – 2010.

In the U.S. there has been a steady rise in the rate of primary thyroid malignancies with 37,200 new cases diagnosed in 2009, 48,000 new cases diagnosed in 2011 and approximately 60,220 new cases diagnosed in 2013.<sup>6-9</sup> There has been increased utilization of fine needle aspirate biopsies (FNAB) in managing these thyroid nodules and in 2011 an estimated 450,000 thyroid fine needle aspirate biopsies (FNABs) were performed.<sup>9,10</sup> Increased incidence of thyroid cancer has been largely attributed to increased use of ultrasound which can detect sub-centimeter thyroid nodules and routine use of FNAB as first line replacing nuclear medicine scans.<sup>4,10,11</sup> The majority of these

new case are very early stage disease, with 68% being T1 cancers of 2.0 cm or less (39% less than 1.0 cm and 29% between 1.0 to 2 cm).<sup>6,10,12</sup> Other hypotheses for this dramatic rise in incidence include increased exposure to medical radiation, increased dietary iodine intake, and increased prevalence of chronic autoimmune thyroiditis.<sup>4,11</sup> Despite the increasing incidence of thyroid cancer, mortality rates have not changed over the past decade, implying that the majority of these newly identified cases are low risk disease.<sup>6,10</sup>

Thyroid neoplasms include thyroid adenoma, well differentiated thyroid cancer, poorly differentiated thyroid cancer, anaplastic (undifferentiated) thyroid cancer and medullary cancer (Figure 2)<sup>13</sup>. Well differentiated thyroid cancer is by far the most common type of thyroid cancer with PTC being the most common in about 85% of the cases, follicular thyroid cancer (FTC) in about 10% of the cases and finally Hurthle cell or oxyphil tumors in 3% of the cases (Table 1).<sup>14,15</sup>

## 1.1 Contemporary Thyroid Nodule Management

According to American Thyroid Association guideline, a detailed history and physical is the first step in evaluating a patient presenting with thyroid nodule (Figure 3).<sup>16</sup> Risk factors for thyroid cancer include family history of thyroid cancer in one or more first degree relatives, certain thyroid cancer syndromes (e.g. Cowden's syndrome, Familial Polyposis, Carney Complex, Multiple Endocrine Neoplasia 2, Werner syndrome) in first degree relative, and previous exposure of neck to ionizing radiation. Thyroid Stimulating Hormone (TSH) levels should be measured, and in patients with low TSH, a radioactive iodine scan should be done to assess for a hyper-functioning nodule. Patients with hyper-

functioning nodules are very unlikely to have a thyroid cancer (~1%). If the patient has a normal or high TSH, ultrasound assessment of the thyroid bed is essential to measure the nodule size and characteristics. Although no single sonographic feature or combination of features is highly specific or sensitive in diagnosing nodules to be malignant, certain ultrasound characteristics, such as microcalcifications, hypoechogenicity compared to the thyroid bed, increased nodular vascularity, infiltrative margins (absent halo) or taller than wide on transverse view, can be helpful in identifying high risk nodules.<sup>17-22</sup> In contrast, spongiform and purely cystic nodules are likely to be benign nodules.<sup>18,23</sup> Ultrasound is also helpful in assessing the presence of any cervical lymphadenopathy in the setting of thyroid cancer, as it identifies suspicious cervical lymphadenopathy in 20 – 31% of the cases.<sup>24-27</sup> For those patients who do not fall into the high risk category, thyroid nodules greater than 1 cm or those which are increasing in size (20% increase in nodule diameter with a minimum increase in two or more dimensions of at least 2 mm or 50% increase in nodule volume) should be biopsied with a fine needle aspirate biopsy (FNAB).<sup>16,28-30</sup> Nodules that are greater than 4 cm should be removed surgically, as the diagnostic sensitivity decreases with increasing size.<sup>31</sup>

FNAB reports are based on the Bethesda system, where the FNAB specimen is assessed by a cytopathologist and the likelihood of malignancy is reported. The results are divided into 5 categories (Table 2).<sup>32</sup> As evident from the malignancy risk, FNAB is extremely useful for the low and high risk categories, but it does poorly in the indeterminate/intermediate category. Thus, patients may undergo diagnostic hemithyroidectomy, surveillance ultrasounds and FNAB, which results in significant patient discomfort and increased cost to the health care system.

## 1.2 Risks of Thyroid Surgery

As previously noted, diagnostic hemi-thyroidectomy is recommended for those with FNAB reported as indeterminate/intermediate risk of cancer. Thyroid surgery is associated with the risks of general anesthetic, bleeding, infection, transient or permanent hypothyroidism, transient or permanent hypocalcemia (if total thyroidectomy), and injury to both superior and recurrent laryngeal nerve resulting in voice alteration, dysphagia and potential airway compromise. Injury to the superior laryngeal nerve can result in inability to raise the pitch of the voice and altered sensation of the hemi-larynx. Recurrent laryngeal injury is more severe, as it results in a paralyzed vocal cord on the affected side, thus resulting in a breathy voice. The overall incidence of this complication is 2 – 10%.<sup>33,34</sup> Although the risks are generally lower in the hands of high volume thyroid surgeons, 50% of thyroid surgeries in the U.S. are performed by low volume surgeons who do less than 5 thyroidectomies per year.<sup>35</sup>

Thus, by improving the accuracy of diagnosis and decreasing the risk of malignancy in the indeterminate/intermediate category of FNAB, diagnostic surgeries can be avoided. This has the potential to decrease costs in an already strained Canadian healthcare system.



### 1.3 Thyroid Cancer Follow up

Currently thyroid cancer surveillance is done by stimulated and unstimulated thyroglobulin levels and radioactive I<sup>131</sup> scan in select cases that receive adjuvant radioactive iodine.<sup>16</sup> Twenty-three to 29% of patients with well differentiated thyroid cancers have antithyroglobulin antibodies, thus making surveillance of thyroid cancer recurrence difficult.<sup>36-39</sup> Moreover, 12% of cases are thyroglobulin negative pre-operatively with the entire gland *in situ*, highlighting the imperfections of thyroglobulin for monitoring disease burden.<sup>40</sup> Thus a new test that can aid in thyroid cancer surveillance would be highly beneficial.

### 1.4 Genetics of Thyroid Cancer

Three distinct pathways are proposed for neoplastic transformation and proliferation of thyroid follicular cells: hyper-functioning follicular thyroid adenoma, FTC and PTC (see Figure 4).<sup>15</sup> Most poorly differentiated and undifferentiated thyroid carcinomas are thought to arise from pre-existing well-differentiated thyroid carcinomas through additional genetic events. Several genes have been implicated in the pathogenesis of thyroid cancer which include: point mutations in *BRAF*, *RAS*, *CTNNB1*, and *TP53*; and gene rearrangement in *BRAF*, *RET*, *NTRK1*, and *PPARG* (Table 3).<sup>15</sup> *BRAF(V600E)* mutation is the most commonly encountered point mutation in PTCs and has been reported in varying percentages from 29 – 69%.<sup>41-49</sup> A recent report by The Cancer Genome Atlas Research Network (TCGA) has further clarified the genomic landscape of

PTCs.<sup>41</sup> Majority of the PTCs appear to be driven by a single mutually exclusive point mutation (most commonly either *BRAF* or *RAS*) or gene fusions. Additionally, PTCs can be subclassified into classical *V600E BRAF-like* PTCs and *RAS-like* PTCs. Thyroid samples were ranked by *BRAF(V600E) – RAS* score (BRS), with BRS being strongly associated with driver mutation status, thyroid differentiation score, histology and follicular fraction. Differential activation of molecular pathways depending on the driver mutations was thought to be the underlying mechanism – classical PTCs were more *BRAF(V600E)-like* on the thyroid differentiation score whereas the histological variants of follicular, tall cell and other variants of PTCs were more *RAS-like* on the thyroid differentiation score.

Thus a test that can help differentiate between a malignant thyroid nodule from a benign one, predict tumor aggressiveness and facilitate cancer surveillance would be extremely helpful. Currently, there are two proprietary tests that are available in United States targeting the intermediate and indeterminate Bethesda categories through molecular testing of additional FNA samples– Afirma and Thyroseq v2. Afirma gene expression classifier developed by Veracyte Inc. (South San Francisco, CA), costs USD \$4200 per nodule and requires samples to be sent to a central facility. This is a “rule out” test with preliminary studies suggesting a high negative predictive value of 94 – 95%, but low positive predictive value of 15 – 37% in the indeterminate category.<sup>50,51</sup> However, more recent follow up studies suggest that the test is less accurate than originally reported.<sup>51-59</sup> Ongoing surveillance with US and US-FNAB is still recommended if the test is negative. Thyroseq v2 offered by CBLPATH and developed by the University of Pittsburgh Medical

Center Division of Molecular and Genomic Pathology costs USD \$2250 and requires samples to be sent to a central facility. Nikiforov published a recent series of 143 patients with FNAB diagnosis of follicular neoplasm (FN)/suspicious for follicular neoplasm (SFN).<sup>60</sup> Thyroseq v2 has been termed as “rule in” test with a high negative predictive value of 96% and a positive predictive value of 83%. Both of these tests are currently not available in Canada, require shipping the samples to a central facility and cannot be used for thyroid cancer surveillance. Typically, additional FNA samples are needed to carry out these tests, which can cause additional patient pain and anxiety.

We propose an alternative test to assess the presence of thyroid cancer using circulating tumor DNA (ctDNA) in plasma samples. Plasma samples provide several advantages over the traditional FNAB or Affirma – there would be significantly less discomfort to the patient with a routine blood draw rather than ultrasound guided FNAB, plasma samples are easy to handle and most tertiary care hospitals already have a polymerase chain reaction (PCR) machines available.

## 1.5 Circulating Tumor DNA (ctDNA)

Cell-free nucleic acids (cfDNA) in human blood were first described in 1948 by Mandel and Metais.<sup>61</sup> There was very little enthusiasm in cfDNA until in 1994 it was discovered that mutated RAS gene fragments in blood were from cancer cells.<sup>62</sup> With recent advances in molecular technology there has been great interest in cfNA in detection and surveillance of cancer (Figure 5 and Figure 6).

cfNA is comprised of DNA, RNA and microRNAs in the plasma or serum and can be used as “liquid biopsy”, circumventing the need for tissue biopsy and facilitating surveillance of cancer. ctDNA can be released by apoptotic and necrotic cancer cells, actively secreted by tumor cells or after tumor cells are processed by macrophages (Figure 7).<sup>63-65</sup> Although the half-life of the ctDNA is variable and estimated to be between 15 minutes to several hours<sup>66,67</sup>, there is constant release of tumor ctDNA. Diehl et al. estimated that for a tumor that weighs 100g (approximately  $3 \times 10^{10}$  tumor cells), up to 3.3% of tumor DNA enters plasma each day.<sup>68</sup>

Analysis of ctDNA has several advantages. These include characterization of molecular profiles when tissue is not available, reflects tumor heterogeneity, allows monitoring of response to therapy, detect residual disease following therapy, assesses tumor evolution with therapy and finally allows analysis of pharmacodynamics of the chemotherapy drugs (Table 4). Thus genetic alterations in ctDNA are better poised as biomarkers than conventional proteins such as CEA or CA19-9, which are expressed both in tumor and normal cells.

In other cancers, ctDNA levels have been shown to be higher in patients with cancer<sup>66,69</sup> and decrease after surgery<sup>70</sup>. However they can also be elevated in non-cancer patients with some overlap in the ranges.<sup>66,71</sup> Importantly, there can be considerable variation amongst different tumor types.<sup>69</sup> This underscores the importance of studying ctDNA in each specific cancer.

ctDNA, which includes coding and noncoding genomic DNA, does allow for monitoring of tumor specific changes and allows detection of genetic and epigenetic changes within the patient. Microsatellite instability, loss of heterozygosity, mutations, polymorphisms, methylation and integrity can be assessed in ctDNA samples. These have been extensively studied in various tumor types including bladder, breast, cervical, colorectal, hepatocellular carcinoma, lung, non-hodgkin's lymphoma, melanoma, ovarian, pancreatic and prostate cancers.<sup>reviewed in 72</sup>

ctDNA has been used for prognostication purposes. *KRAS* hotspot mutations and *CDKN2A* hypermethylation in patients with colorectal cancer was investigated by Lecomte et al. The 2-year survival rate was 100% in patients who did not *KRAS* mutations or *CDKN2A* gene promoter hypermethylation.<sup>73</sup> In addition patients who had residual detectable ctDNA after surgery relapsed within 1 year.<sup>68</sup>

Tumor heterogeneity is a recognized pitfall of tumor tissue sampling, with detection of ctDNA providing an avenue to potentially mitigate this. Kuo et al. showed that ctDNA provides a better representation of the overall cancer than focal primary site tumor biopsies.<sup>74</sup>

ctDNA can be used as a predictive biomarker to monitor response to therapy as well as for prognostication. Tie et al. found that in metastatic colorectal cancer, ctDNA was detectable in plasma and early changes in ctDNA during chemotherapy predicted the radiologic response, which was seen later in the time course.<sup>75</sup> Bettegowda et al. in 31

patients with colorectal cancer found 70 somatic mutations that were not detected in tumor or plasma before initiation of EGFR blockade.<sup>69</sup> They also found that colorectal tumor patients who had metastatic disease with low levels of ctDNA lived significantly longer than patients with higher levels. A similar association has been reported in breast cancer patients as well.<sup>76</sup>

Following the amount of ctDNA and the genetic alterations of ctDNA over time can enable real time surveillance of cancer, early detection and guide therapeutic management. Additionally, ctDNA can theoretically account for genetic heterogeneity within the tumor. Other important advantage is that plasma is easier to collect and process, and health laboratories in most hospitals have readily available technology for molecular processing. Role of ctDNA has not been thoroughly investigated in thyroid cancer.

## 1.6 Hypothesis

PTC nodules harboring *BRAF(V600E)* ctDNA will shed *BRAF(V600E)* ctDNA in blood.

Thus *BRAF(V600E)* ctDNA can be used to differentiate between PTC and benign nodules.

## 1.7 Objectives

1. Detect *BRAF(V600E)* ctDNA in preoperative plasma samples of patients undergoing thyroid surgery for nodular thyroid disease.
2. Compare *BRAF(V600E)* ctDNA levels in preoperative and postoperative plasma samples of patients undergoing thyroid surgery for nodular thyroid disease.
3. Determine concordance of *BRAF(V600E)* between preoperative plasma samples and the corresponding FFPE sample of index thyroid nodule.

## 2 Methods

### 2.1 Patient Recruitment

Patients referred to the Otolaryngology – Head and Neck Surgery Clinic at the London Health Sciences Centre for thyroid nodules from April 2014 to March 2015 were approached for participation in the study.

#### 2.1.1 Eligibility

Inclusion criteria included patients over the age of 18 and those scheduled to undergo partial or total thyroidectomy for their thyroid nodules.

Exclusion criteria included previous cancer that is known to be positive for *BRAF(V600E)* mutation (such as melanoma, lung cancer and colon cancer).

#### 2.1.2 Ethics Board Approval

Patients were recruited as per the London Health Sciences Center (LHSC) Research Ethics Board Approval (see Appendix D, Appendix E, Appendix F and Appendix G).



### 2.1.3 Sources of Funding

This study was supported by a grant from The Thyroid Cancer Foundation – London Chapter and internal funding from the Department of Otolaryngology – Head and Neck Surgery.

## 2.2 Specimen Collection

Patient's blood was collected in 5 mL EDTA coated blood collection tubes by the LHSC lab. This was then promptly transferred to our lab and blood was processed. Blood was separated into plasma and red blood cells by centrifuging at 1000g for 10 minutes at room temperature. Plasma was then pipetted out and aliquoted in 1 ml cryovials and frozen at -80°C.

## 2.3 Isolation of Circulating Nucleic Acids

Previously processed and frozen 1 ml aliquots of plasma samples were thawed and equilibrated to room temperature. QIAamp circulating nucleic acid kit (Cat no. 55114) was used using a vacuum manifold for isolation of circulating nucleic acids. 100 µl of Proteinase K was pipetted in a 50 ml centrifuge tube. 1 ml of plasma was added into the tube. 0.8 ml of Buffer ACL (containing 1 µg carrier RNA) was added and the mixture was vortexed and incubated at 60°C for 30 min. 1.8 ml of Buffer ACB was added to the

lysate and vortexed. The lysate-Buffer ACB mixture was incubated on ice for 5 mins. The mixture was then applied into the QIAamp Mini Column placed in the vacuum manifold. Once the mixture was completely through the column, 600 ul of Buffer ACW1 was then applied. Once Buffer ACW1 was completely through the column, 750 ul of Buffer ACW2 was then applied. Once Buffer ACW2 was completely through the column, 750 ul of ethanol was then applied. The column was then removed from the vacuum manifold and placed in a collection tube and centrifuged at 14,000 rpm for 3 min. The column was placed in a new collection tube and incubated at 56°C for 10 min to dry the membrane. The column was then placed in new elution tube and 100 ul of Buffer AVE was applied to the center of the membrane and incubated at room temperature for 3 mins. Thereafter, the column was centrifuged for 1 min at 21,000g to elute the nucleic acids. Concentration of the nucleic acids was measured using a NanoDrop spectrophotometer.

## 2.4 Isolation Of Nucleic Acids From Formalin Fixed Paraffin Embedded (FFPE) Samples

Index thyroid nodules were identified on the paraffin embedded post-surgical specimens. Cores were obtained and nucleic acids were extracted using the QIAamp DNA FFPE tissue kit (Cat no. 56404). 1 ml xylene was added to the core FFPE specimens and the microcentrifuge tube was vortexed and then centrifuges at 14,000 rpm for 2 min. The supernatant was carefully removed and 1 ml of ethanol was added to the pellet. The mixture was vortexed and then centrifuged at 21,000g for 2 min. The tube was then

incubated at 37°C until the residual ethanol had evaporated. The pellet was then resuspended in 180 ul Buffer ATL followed by addition of 20 ul of proteinase K and mixed by vortexing. The samples were then incubated at 56°C for at least 1 hr (or until the sample had been completely lysed). Thereafter the samples were incubated at 90°C for 1 hr. The samples were then briefly centrifuged and 200 ul of Buffer AL was added followed by addition of 200 ul of ethanol. After brief centrifugation, the entire lysate was transferred to a QIAamp MinElute column and centrifuged at 7,000g for 1 min. The column was placed in a clean collection tube and centrifuged at 21,000g for 3 min. The column was placed in a clean microcentrifuge tube and 100 ul of Buffer ATE was applied for elution and it was incubated for 5 min. The column was then centrifuged at 21,000g for 1 min. Concentration of the nucleic acid was measured using a NanoDrop spectrophotometer.

## 2.5 Quantitative Polymerase Chain Reaction (qPCR) for *BRAF(V600E)*

The Qiagen QuantiTect Multiplex PCR Kit (Cat No. 204543) was used for qPCR. Briefly, a 20 ul reaction with 2X QuantiTect Multiplex PCR Master Mix, 4 uM (0.04 ul of 100 uM) forward primer, 4 uM (0.04 ul of 100 uM) reverse primer, 4 uM (0.04 ul of 100 uM) probe were used. RNase-free water was used to bring the final volume of each reaction to 20 ul. 0.2 ul of DNA template was used in each 20 ul reaction. See Table 5 for reaction setup. Each reaction included primer-probe sets specific for wildtype sequence of the *BRAF* gene (this sequence was in an exon that is not known to harbor any

mutations) and the *BRAF(V600E)* mutation (Figure 8 and Table 6). Table 7 outlines the cycling conditions.

The presence of wild type *BRAF* was used as an internal control for the qPCR reaction. Dilutions of 1:1, 1:10, 1:100, 1:250 of the BHT-101 cell line were used for standard curve generation. ASH-3 cell line was used as negative control. Baseline was determined based on the negative control. Cycle threshold (Ct) was determined where the amplification plot crosses the baseline. Each sample was done in duplicates – amplification plots of duplicates were averaged and a single curve was generated to determine the Ct (Figure 9). Each sample was repeated at least twice. Ct for each sample was then used to determine the amount of *BRAF* and *BRAF(V600E)* mutation using the standard curve (Figure 10). The relative amount of *BRAF(V600E)* was calculated by the following formula:  $BRAF(V600E) / BRAF\ WT \% = [V600E] / [WT] \times 100$ . Selected samples which were positive and negative for *BRAF(V600E)* mutation were sent for Sanger sequencing to confirm the qPCR findings.

## 2.6 Statistical Analysis

Statistical Analysis was done using SPSS. Student's t-test was done to compare pre-operative and post-operative *BRAF(V600E)* ctDNA. Fischer's exact test was used to determine the association between detectable *BRAF(V600E)* ctDNA in preoperative plasma samples and pathologic characteristics.

## 3 Results

### 3.1 Patient Characteristics

A total of 61 patients were recruited. 55 (90.2%) patients were being considered for surgery and six (9.8%) patients were being considered for iodine radiation therapy for locoregional recurrence and were being considered for iodine radiation therapy. 21 (34.4%) were males and 40 (65.6%) were females. Of those 55 patients who underwent surgery, final pathology of the thyroid nodules showed 13 (21.3%) were benign, 26 (42.6%) were classical PTC, 13 (21.3%) were non-classical PTC, 3 (4.9%) were FTC. See Table 8 for baseline patient characteristics and final pathology of the index nodule.

Of those 55 patients with preoperative samples and being considered for surgery, postoperative samples were obtained from 37 patients (67.3%). 12 (32.4%) were males and 26 (67.6%) were females. Final pathology of the corresponding index thyroid nodule showed 9 (21.6%) were nodular hyperplasia, 18 (48.6%) were classical PTC, 8 (21.6%) were non-classical PTC, 3 (8.1%) were FTC. See Table 9 for paired preoperative and postoperative patient characteristics and final pathology of the index nodule.

### 3.2 *BRAF(V600E)* detection in preoperative samples in patients with thyroid nodules

A total of 9/61 (14.8%) patients had detectable *BRAF(V600E)* in the preoperative plasma samples (Figure 11). In patients with final diagnosis of nodular hyperplasia, 0/13 (0%) patients had detectable *BRAF(V600E)* ctDNA in the preoperative plasma samples. In patients with final diagnosis of classical PTC, 8/26 (30.8%) patients (patients 1, 6, 39, 40, 41, 42, 57, 63) had detectable *BRAF(V600E)* ctDNA in the preoperative plasma samples. In patients who had undergone previous thyroidectomy and had a metastatic locoregional recurrence of classical PTC, 0/2 (0%) patients had detectable *BRAF(V600E)* ctDNA in the preoperative plasma samples. In patients who had undergone previous thyroidectomy and had a metastatic locoregional recurrence of non-classical PTC, 1/4 (25%) patients (patient 26) had detectable *BRAF(V600E)* ctDNA in the preoperative plasma samples.

### 3.3 Ability of *BRAF(V600E)* ctDNA to detect classical papillary thyroid cancer

Using the pathologic diagnosis of the index thyroid nodule as the standard, presence of *BRAF(V600E)* ctDNA in preoperative plasma samples was used to differentiate between classical PTCs and benign thyroid nodules (Table 10). All the patients with detectable *BRAF(V600E)* ctDNA had classical PTCs and none of the benign thyroid nodules had detectable *BRAF(V600E)* ctDNA. This was statistically significant ( $p < 0.05$ ).

### 3.4 *BRAF(V600E)* detection before and after surgery in patients with thyroid nodules

37 patients were followed post-operatively with an additional blood draw at one month follow up. 6/37 (16.2%) patients had detectable *BRAF(V600E)* ctDNA in preoperative plasma samples and all of them decreased postoperatively (p-value < 0.05), with five patients having non-detectable *BRAF(V600E)* postoperatively (Figure 12).

The results were further analyzed based on the final histology of the index thyroid nodule. None of the patients 0/8 (0%) with a final diagnosis of nodular hyperplasia had detectable *BRAF(V600E)* ctDNA (Figure 13). 6/18 (33.3%) patients with a final diagnosis of classical PTC had detectable *BRAF(V600E)* ctDNA and they all decreased post-operatively (Figure 14) (p-value < 0.05). None of the patients with final diagnosis of non-classical PTC - 0/8 (0%) (Figure 15) and FTC - 0/3 (0%) (Figure 16) had detectable *BRAF(V600E)* ctDNA.

### 3.5 Correlation of *BRAF(V600E)* ctDNA with classical PTC thyroid cancer staging

5/6 (83.3%) patients with detectable preoperative *BRAF(V600E)* ctDNA and final diagnosis of classical PTC had higher T-stage (T3) compared to 1/6 (16.7%) patient had a lower T-stage disease. This was not statistically significant (p-value > 0.05) (Table 11). No association was noted with detectable levels of *BRAF(V600E)* ctDNA and nodal metastases and extra-thyroidal extension (ETE) (p > 0.05).

### 3.6 Detection of *BRAF(V600E)* in FFPE samples and concordance with the presence of *BRAF(V600E)* ctDNA in preoperative plasma samples

To assess the concordance between preoperative *BRAF(V600E)* ctDNA and *BRAF(V600E)* mutational status of the index thyroid nodule, FFPE samples were obtained and mutational status for *BRAF(V600E)* was determined (see Table 12). In patients with final diagnosis of nodular hyperplasia: 8/8 (100%) did not have *BRAF(V600E)* mutation in both FFPE and preoperative ctDNA samples. In patients with final diagnosis of classical PTC: 4/18 (22.2%) patients had detectable *BRAF(V600E)* mutation in both FFPE and preoperative plasma ctDNA samples (see Figure 17), 3/18 (16.7%) did not have *BRAF(V600E)* mutation in both FFPE and preoperative ctDNA samples, 9/18 (50%) patients had *BRAF(V600E)* mutation in FFPE sample but not in the preoperative plasma sample, and 2/18 (11.1%) patient had *BRAF(V600E)* mutation in preoperative ctDNA plasma sample but not in the FFPE sample.



## 4 Discussion

The results of this study indicate that ctDNA may have a role in diagnosing thyroid cancer and its surveillance. US-FNAB is highly accurate in ruling out thyroid cancer in low risk biopsies and ruling in cancer high risk categories (suspicious and positive for malignancy). The specificity decreases in the indeterminate/intermediate group (Table 2). Tests assessing the molecular profile of the thyroid nodules are available. However, financial considerations as well as the necessity to send samples to a central repository is a major barrier for these tests.<sup>50,60</sup> Additionally patients continue to require follow up US-FNAB on regular intervals. Moreover these tests would not be helpful in tumor surveillance.

*BRAF(V600E)* was chosen for this pilot study as it is the most common genetic alteration noted in PTCs and PTCs is the most common type of well-differentiated thyroid cancer.<sup>41</sup> There have been varying reports of detection of *BRAF(V600E)* ctDNA in plasma of patients with thyroid cancer ( Table 14 lists relevant studies). Bettegowda et al. attempted to detect ctDNA in a multitude of tumor types, however only 4 patients in their study (1%) had thyroid cancer.<sup>69</sup> Moreover the mutations detected were all in different genes or codons. Pupilli et al. studied 19 patients with thyroid cancer with pre-operative and post-operative blood testing. They found that 12/17 (71%) of patients had non-detectable *BRAF(V600E)* ctDNA post-operatively and the other 5 patients had detectable levels post-operatively but they were significantly lower compared to the pre-operative levels.<sup>77</sup> Cradic et al. reported *BRAF(V600E)* ctDNA in 20 (11.6%) of the 173 thyroid cancer

patients correlating with the presence of active disease at the time of the blood draw.<sup>78</sup> Chuang et al. found that 3/14 (36%) of patients with PTC had *BRAF(V600E)* ctDNA.<sup>79</sup> Kim et al. found *BRAF(V600E)* ctDNA in only 6.1% of the patients (3/49) with all three patients having lateral lymph node or lung metastasis.<sup>80</sup> Zane et al. reported *BRAF(V600E)* ctDNA in 0% of the patients (0/181) as they were unable to analyze ctDNA.<sup>81</sup>

We successfully detected *BRAF(V600E)* ctDNA in 9/61 (14.8%) patients with thyroid nodular disease selected for surgery. Comparing the paired plasma samples, overall 6/37 (16.2%) had detectable *BRAF(V600E)* ctDNA with significant decline in all six patients post-operatively. 0/8 (0%) patients with nodular hyperplasia had detectable *BRAF(V600E)* preoperative plasma ctDNA, and the levels were non-detectable postoperatively. Based on the molecular profiling reported by the TCGA group, we decided to further classify the PTCs into classical and non-classical PTCs. 6/18 (33.3%) classical PTC patients had detectable *BRAF(V600E)* preoperative plasma ctDNA – with five patients having non-detectable *BRAF(V600E)* ctDNA levels post-operatively, while one of the patients had a significant decline but remained detectable. This patient had undergone total thyroidectomy, right central neck dissection and right neck dissection for this disease. Intra-operatively, recurrent laryngeal nerve was encased in tumor and had positive margins on the final pathology. Additionally, this patient had undergone SPECT CT after his radioactive iodine treatment which showed two focal areas of uptake within the thyroid bed suggesting residual disease. Thus drop in the *BRAF(V600E)* ctDNA levels postoperatively may have an important role in cancer surveillance.

In our study, we found that 0/8 (0%) patients with nodular hyperplasia on final pathology had *BRAF(V600E)* ctDNA. Previous reports have suggested that up to 5.4% of thyroid nodules determined to be benign on FNA can harbor *BRAF(V600E)* mutations, and when combined with 2 or more high risk ultrasound features 88% are malignant on final pathology.<sup>82</sup> It has been speculated that these *BRAF(V600E)* positive “benign” thyroid nodules may in fact be pre-malignant and would eventually turn malignant.<sup>83</sup>

Additionally, although pathology is the gold standard sampling error cannot be discounted. During tissue processing only representative samples are typically obtained especially in larger thyroid nodules and thus it is plausible that foci of cancer maybe missed resulting in under-diagnosis of cancer. Patient 42 was initially diagnosed as benign thyroid nodule on final pathology specimen, however after the study results indicated that the patient had detectable *BRAF(V600E)* ctDNA in preoperative plasma samples and subsequent decline in the levels after surgery, the pathology specimen was reviewed and was diagnosed with classical PTC. This was confirmed with the HMBE-1 immunostaining as well.

Although our results were not statistically significant, there was a trend with correlation of *BRAF(V600E)* ctDNA with T-stage.; no correlation with ETE or nodal metastasis was noted. We speculate that with increased sample size all three of these poor pathologic indicators (T-stage, ETE and nodal metastases) would all be associated with *BRAF(V600E)* ctDNA. *BRAF(V600E)* mutation in PTC has been shown to correlate with poorer prognosis.<sup>84</sup> Tufano et al. included 14 studies in their meta-analysis to assess the

prognosis of PTC in the presence of *BRAF(V600E)*.<sup>85</sup> Risk ratios in *BRAF(V600E)* positive patients were 1.93 for PTC recurrence, 1.32 for lymph node metastasis, 1.71 for ETE, 0.95 for distant metastasis and 1.70 for advanced stage AJCC III/IV. It is conceivable that patients with *BRAF(V600E)* ctDNA can be treated newly developed *BRAF(V600E)* inhibitors such PLX4720, PLX4032 and sorafenib<sup>86-88</sup>.

To assess the utility of *BRAF(V600E)* ctDNA in distinguishing benign versus malignant thyroid pathology, *BRAF* mutational status in the FFPE index thyroid nodule was assessed. 4/6 (66.7%) classical PTC patients who had detectable *BRAF(V600E)* ctDNA also had *BRAF(V600E)* mutation in the FFPE samples and 2/6 (33.3%) patients had FFPE samples with no *BRAF(V600E)* detectable in FFPE but present in ctDNA.. Chuang et al. reported 3/5 (60%) patients who had detectable *BRAF(V600E)* ctDNA compared to the corresponding FFPE tissue.<sup>79</sup> Cradic et al. in their study reported one (11.11%) patient out of nine having detectable *BRAF(V600E)* ctDNA while the corresponding FFPE sample was negative for *BRAF(V600E)*.<sup>78</sup> Although 11 more patients had detectable *BRAF(V600E)* ctDNA their FFPE status was not known. Pupilli et al. also had 3/9 (33.3%) patients who had detectable *BRAF(V600E)* ctDNA but did not have *BRAF(V600E)* mutation in the corresponding FFPE tissue.<sup>77</sup> In contrast to our study, the concordance between detectable *BRAF(V600E)* ctDNA and FFPE *BRAF(V600E)* mutational status was statistically significant.<sup>77</sup> In our study, 2/6 (33.3%) patients with detectable *BRAF(V600E)* in the FFPE samples but not in ctDNA. Sampling error could have affected the inability to detect *BRAF(V600E)* in the FFPE samples, resulting in poor correlation of circulating and primary tumor *BRAF(V600E)* status observed in our study. Thyroid cancer is often a multi-focal disease with tumor heterogeneity. It has been

demonstrated that *BRAF(V600E)* can be acquired as a secondary change during tumor progression or it might be limited to subclone or separate focus in a multifocal tumor.<sup>83,89</sup> As previously mentioned, sampling error was noted in one of our samples; patient 42 who was initially diagnosed with benign nodular hyperplasia on pathology but after detectable *BRAF(V600E)* ctDNA and review of the pathology, a focus of HBME-1 immunostain positive classical PTC was noted. It is also likely that the index nodule may be BRAF mutant but does not happen to shed significant *BRAF(V600E)* DNA due to biological factors including tumor size, invasion and nodal metastases resulting in negative ctDNA levels. Additionally it is important to remember that *BRAF(V600E)* ctDNA can be positive in plasma in other malignancies including melanoma, lung cancer and colorectal cancer.<sup>43,69</sup> The fact that the levels declined to non-detectable levels in our study in that patient suggest that the source of the *BRAF(V600E)* ctDNA was in fact thyroid tissue.

## 5 Conclusion and Future Directions

In summary, our study shows that *BRAF(V600E)* ctDNA can differentiate between malignant classical PTC thyroid cancer and benign nodules. Postoperative decline in *BRAF(V600E)* ctDNA suggests its utility as a potential marker of surveillance marker in a subset of thyroid cancers. Further work is needed to delineate its utility to identify cancers with higher T-stage, ETE and nodal metastasis.

We are continuing to recruit more patients to increase the power of the study. Next generation sequencing (NGS) is a newer technology with ability to detect point

mutations, copy number variations and translocations. Ion-Torrent NGS platform will be used to increase the sensitivity of the detection in. Current study only included *BRAF(V600E)* however a host of additional driver mutations including point mutations, copy number variations and translocations have been identified in thyroid cancer.<sup>41</sup> NGS will provide a platform to include an expanded panel of genes thus increasing the coverage of mutations.

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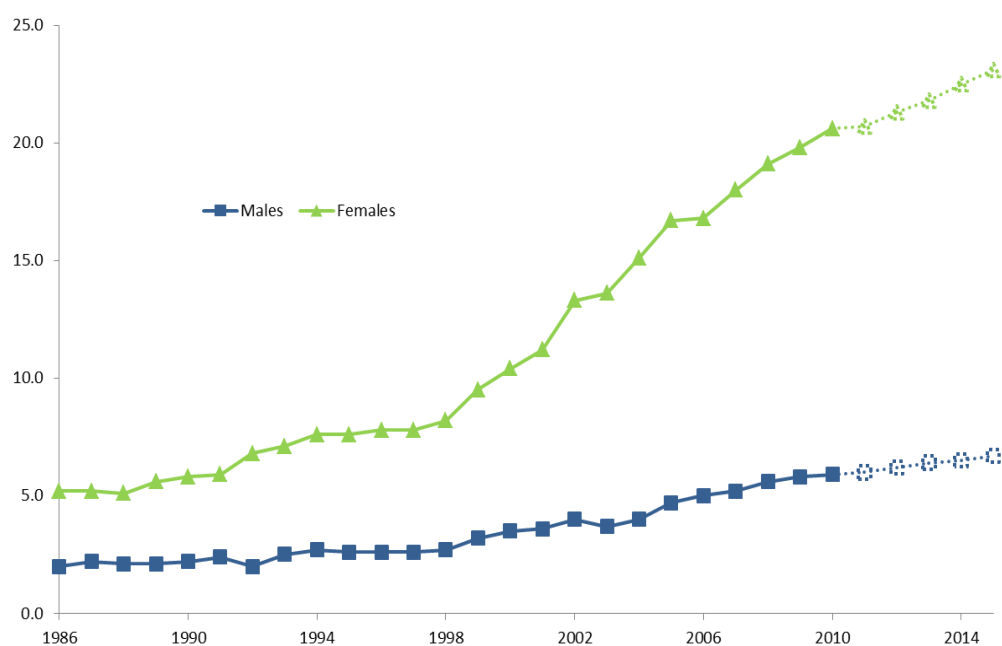
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# Appendices

## Appendix A: Figures

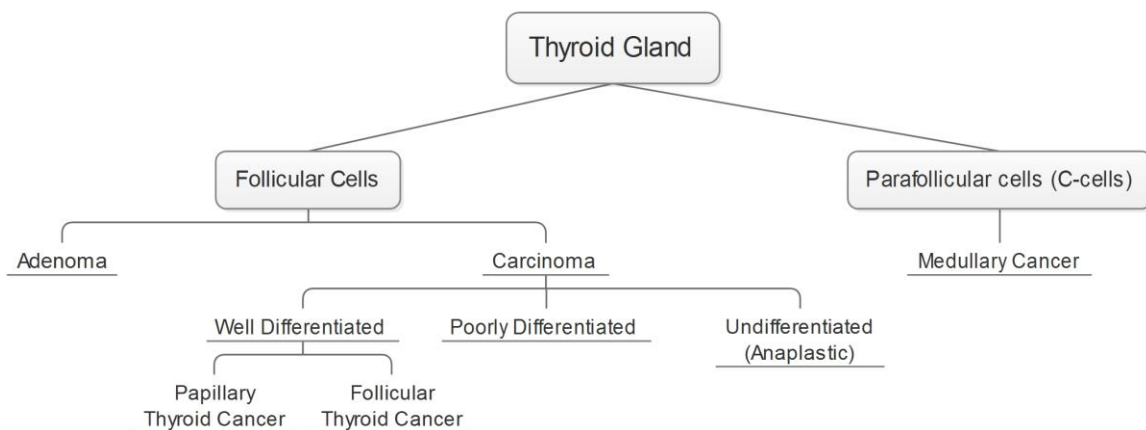
**Figure 1:** Age-standardized incidence rates (ASIR) for thyroid cancer in males and females in Canada, 1986 – 2015. Data from 2011 to 2015, represented by dashed lines is predicted.



Data from Canadian Cancer Society's Advisory Committee on Cancer Statistics<sup>5</sup>



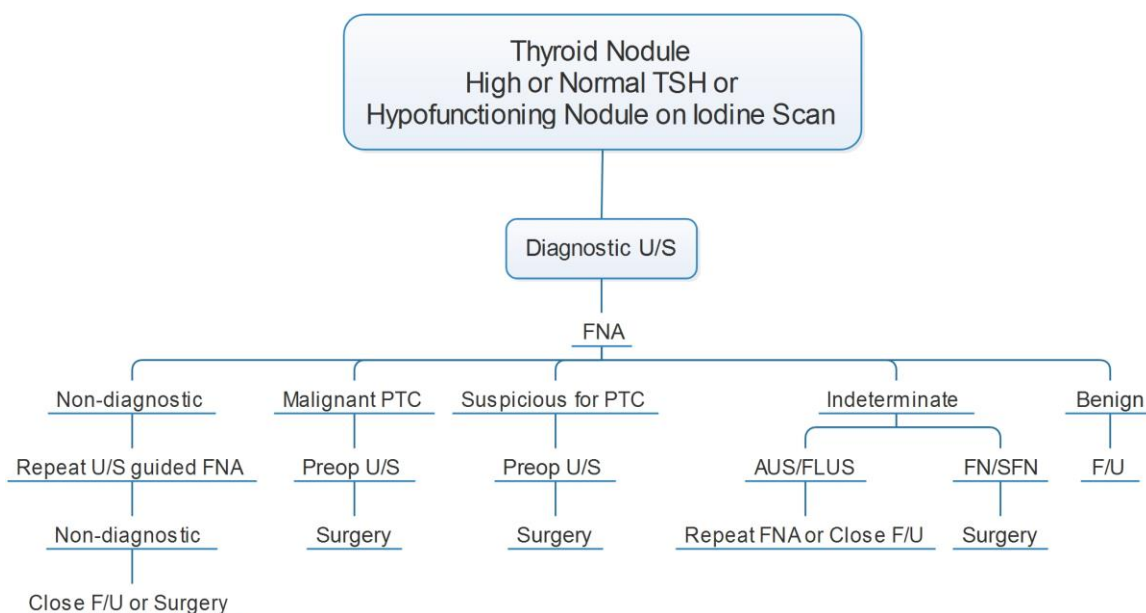
**Figure 2:** Different types of thyroid cancers. Thyroid gland is made of two cell types – follicular cells and parafollicular cells. Majority of the thyroid cancers arise from the follicular cells and papillary thyroid cancer is the most common type of thyroid cancer.



Modified from Caria and Vanni <sup>13</sup>

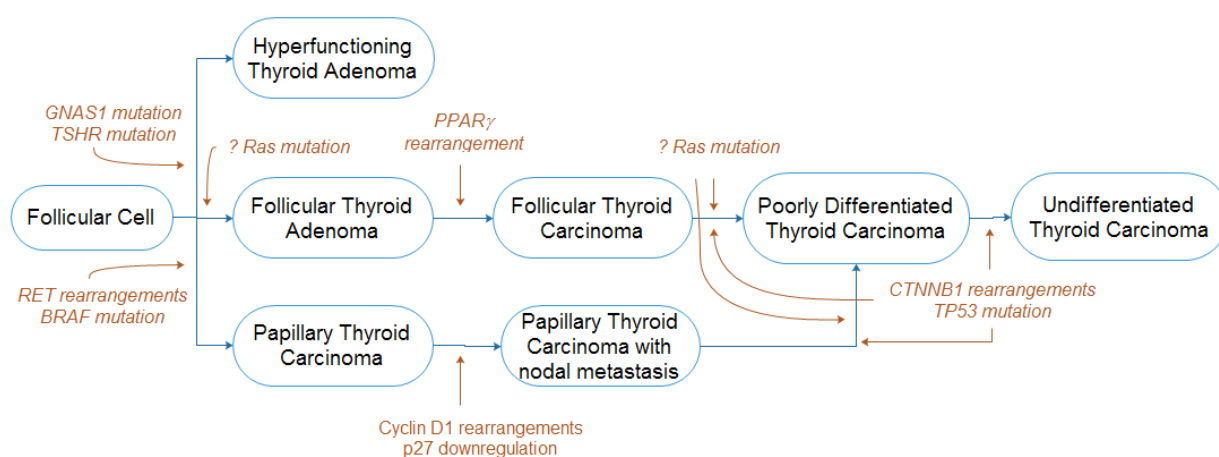
**Figure 3:** Algorithm for the evaluation of patients with one or more thyroid nodules.

Patients initially undergo characterization of the thyroid nodule as well as their thyroid function status. Patients then undergo FNA. Further management of thyroid nodule depends on the results of the FNA.



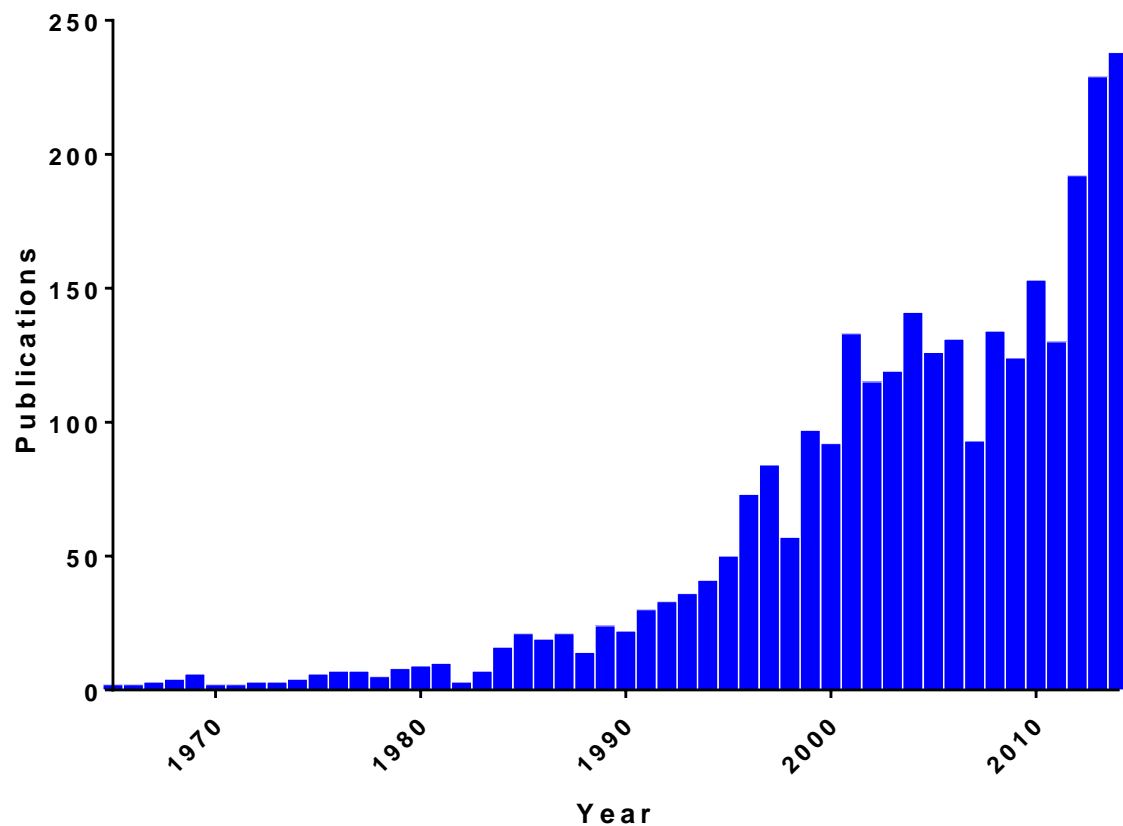
Modified from American Thyroid Association Guidelines Taskforce on Thyroid, et al. <sup>16</sup>

**Figure 4:** Proposed model of thyroid carcinogenesis. Three distinct pathways are proposed for neoplastic transformation and proliferation of thyroid follicular cells: hyperfunctioning follicular thyroid adenoma, FTC and PTC. Most poorly differentiated and undifferentiated thyroid carcinomas are thought to arise from pre-existing well-differentiated thyroid carcinomas through additional genetic events.

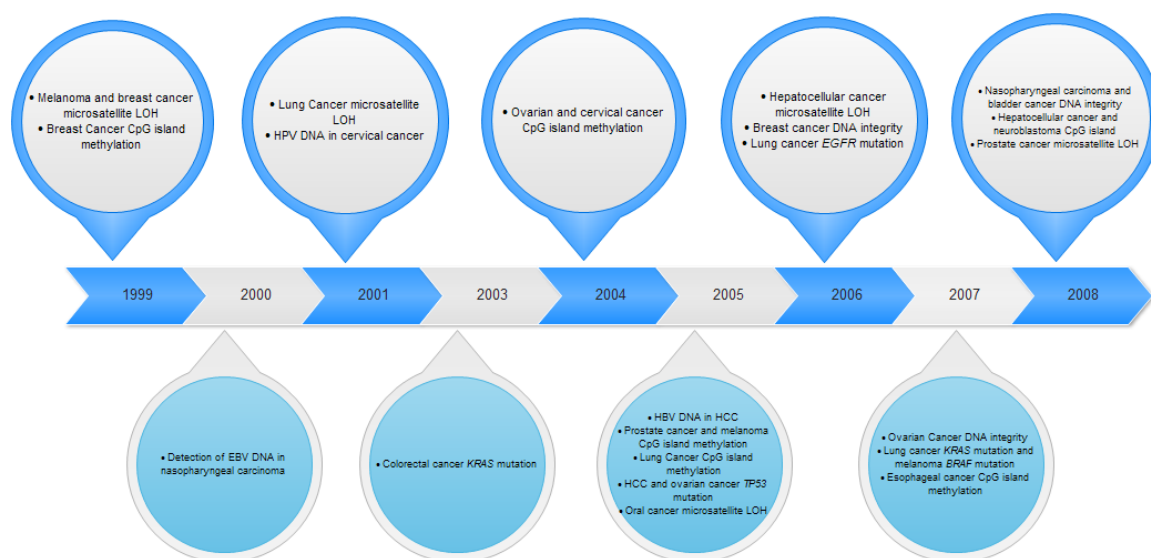


Modified from Kondo, et al. <sup>15</sup>

**Figure 5:** Publications of ctDNA in Pubmed. There has been a recent explosion of publications investigating ctDNA. Y-axis represents number of publications. X-axis represents years.

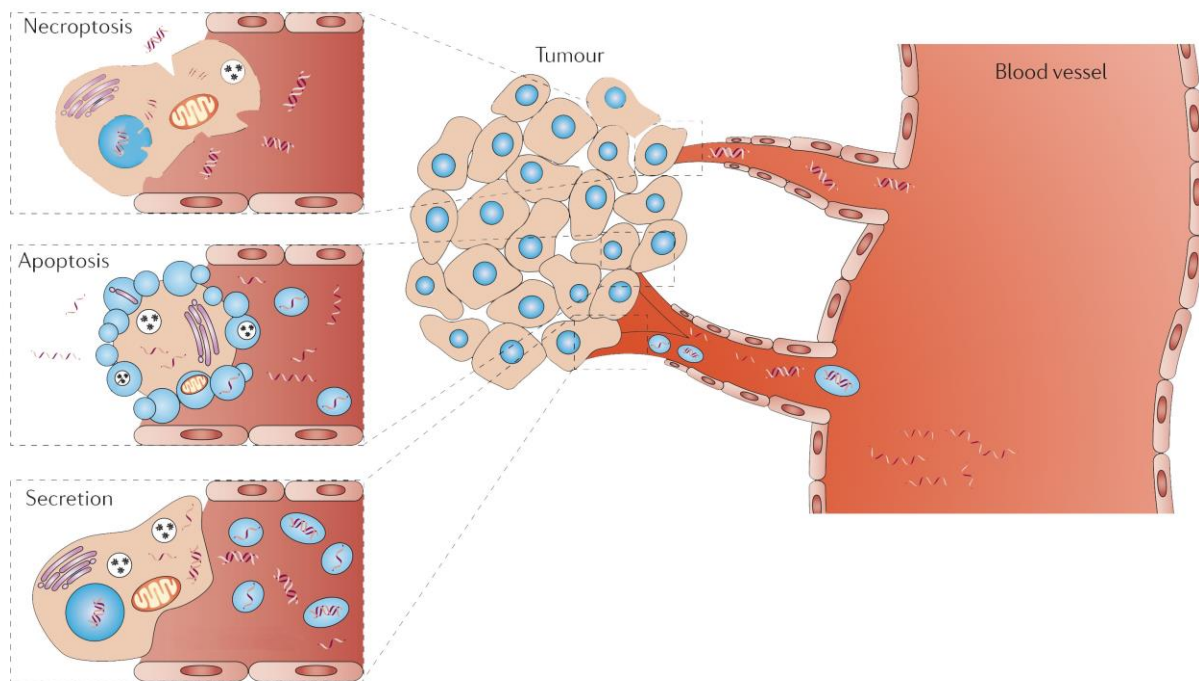


**Figure 6:** Important studies investigating cfDNA.



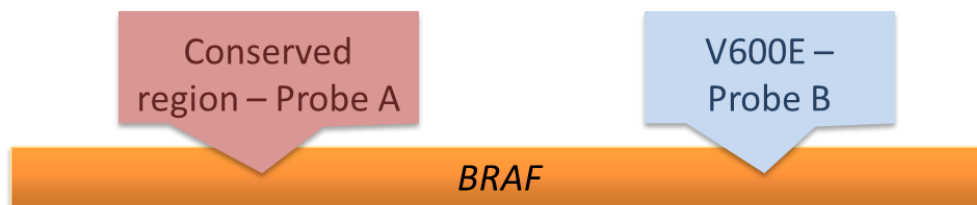
Modified from Schwarzenbach, et al. <sup>72</sup>

**Figure 7:** Sources of cell free nucleic acids include necrosis, apoptosis and secretion by tumor and macrophages. These can then be detected in blood.

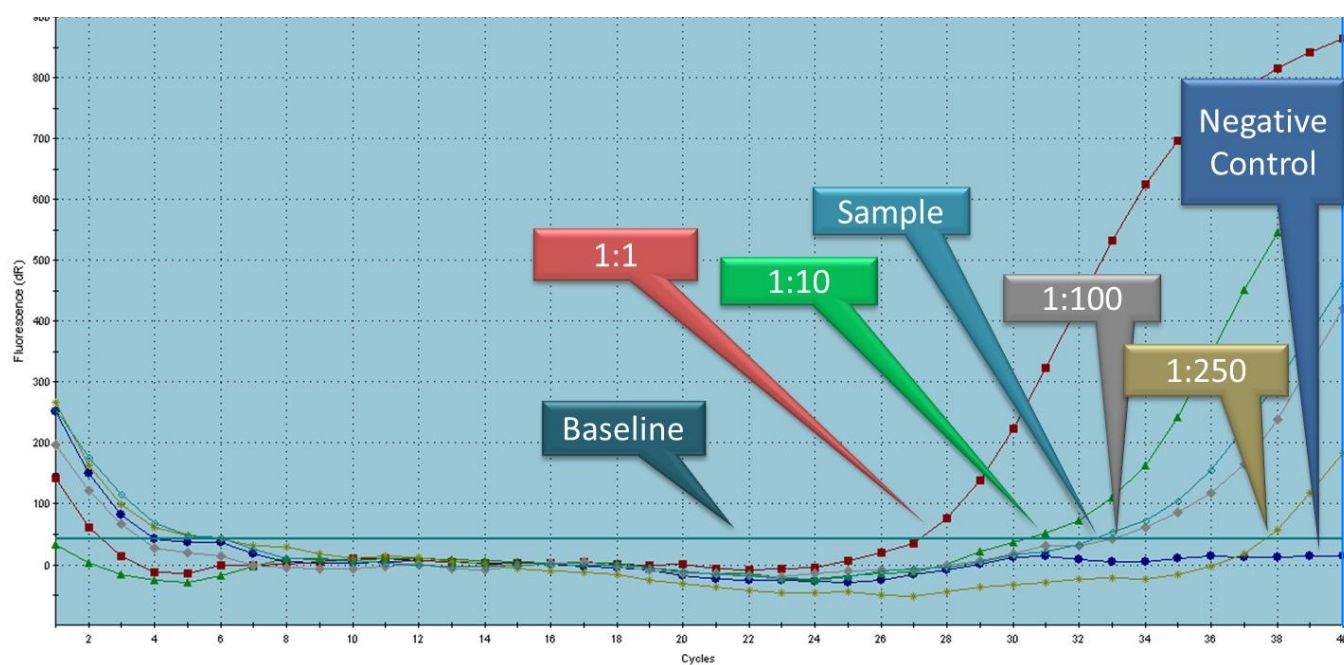


Modified from Schwarzenbach, et al. <sup>72</sup>

**Figure 8:** qPCR primers were designed for non-mutated conserved *BRAF* region and *BRAF(V600E)* mutation. The non-mutated conserved BRAF region was used as an internal control of the qPCR reaction.

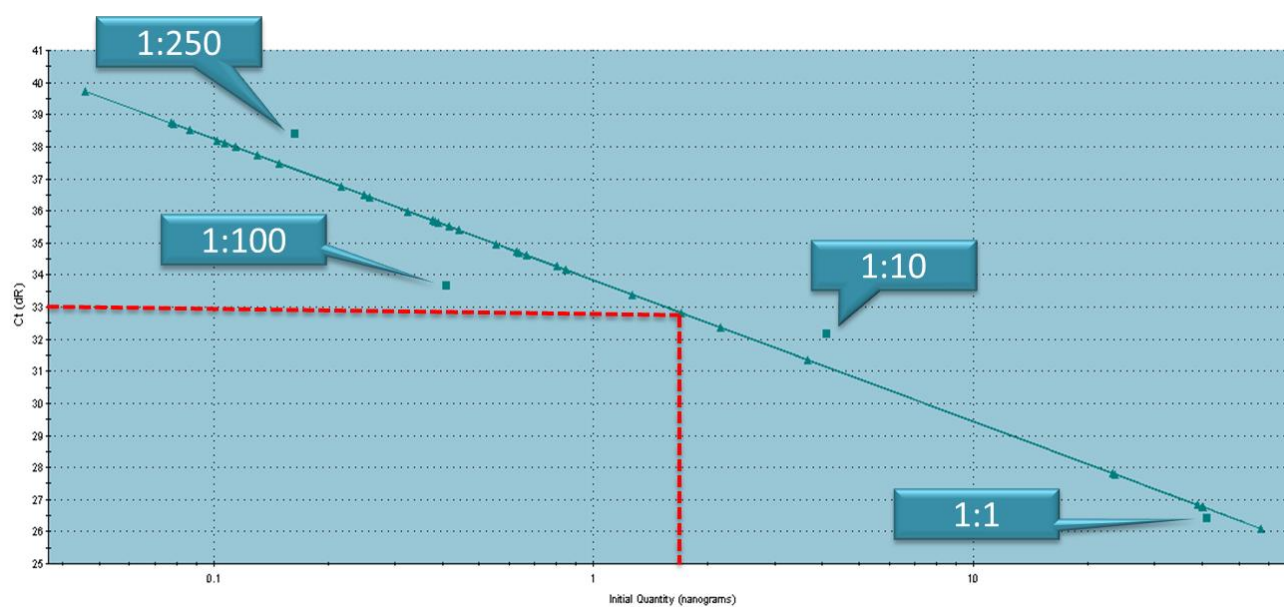


**Figure 9:** Example of qPCR amplification plot of *BRAF(V600E)* with BHT-101 DNA standards and patient ctDNA sample. ASH-3 cell line DNA was used as negative control. Y-axis represents the fluorescence and X-axis represents the number of cycles. Baseline was determined based on the negative control. Ct was determined where the amplification plot crosses the baseline. Each sample was done in duplicates – amplification plots of duplicates were averaged and a single curve was generated to determine the Ct.

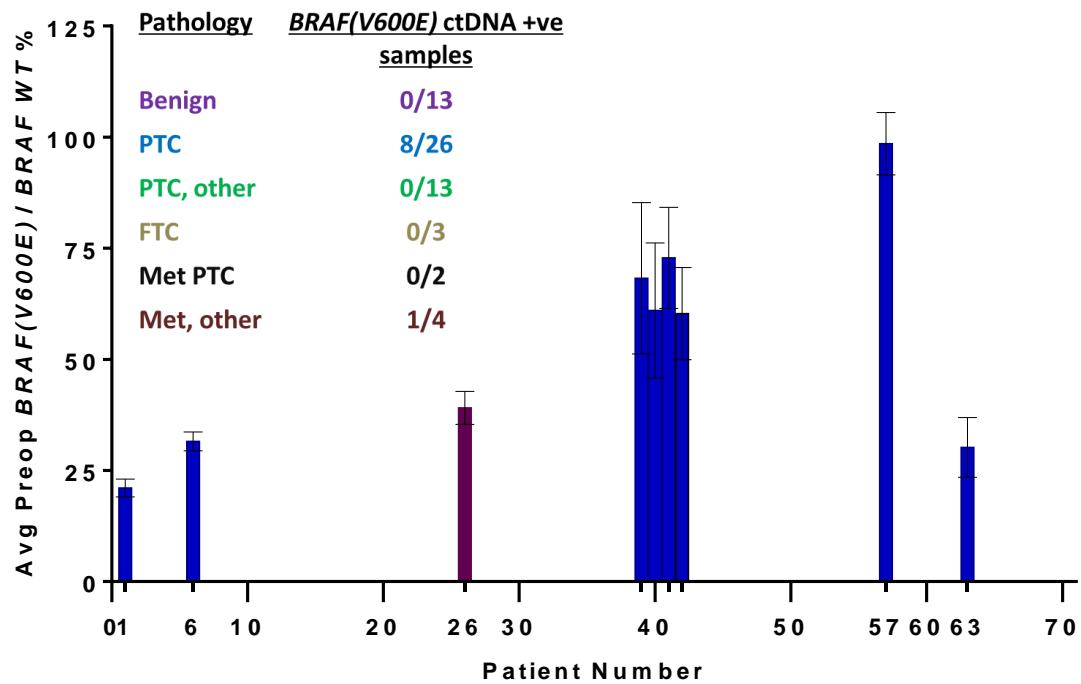




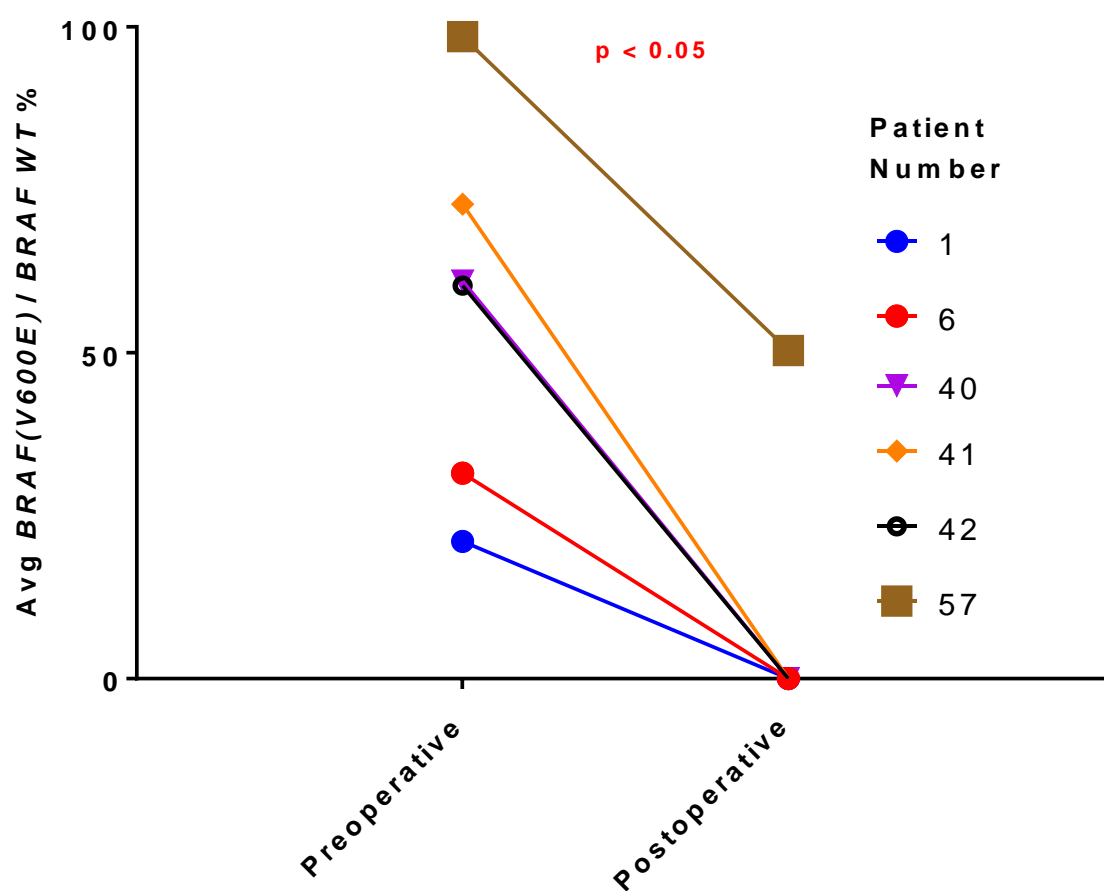
**Figure 10:** Example of standard curve generation from the Ct value obtained from BHT-101 standards. 1:1, 1:10, 1:100 and 1:250 dilution of BHT-101 was used as standard for both non-mutated conserved *BRAF* region and *BRAF(V600E)*. Y-axis represents Ct and X-axis represents amount of DNA in nanograms. The relative amount of *BRAF(V600E)* was calculated by the following formula:  $BRAF(V600E)/BRAF\ WT\ \% = [V600E] / [WT] \times 100$ .



**Figure 11:** Detection of *BRAF(V600E)* in preoperative samples as determined by relative amount of *BRAF(V600E)* (mean  $\pm$ SE). 9/61 (14.8%) patients had detectable *BRAF(V600E)* ctDNA. Y-axis represents average preoperative *BRAF(V600E)* / *BRAF* wildtype percentage. X-axis represents individual patients.

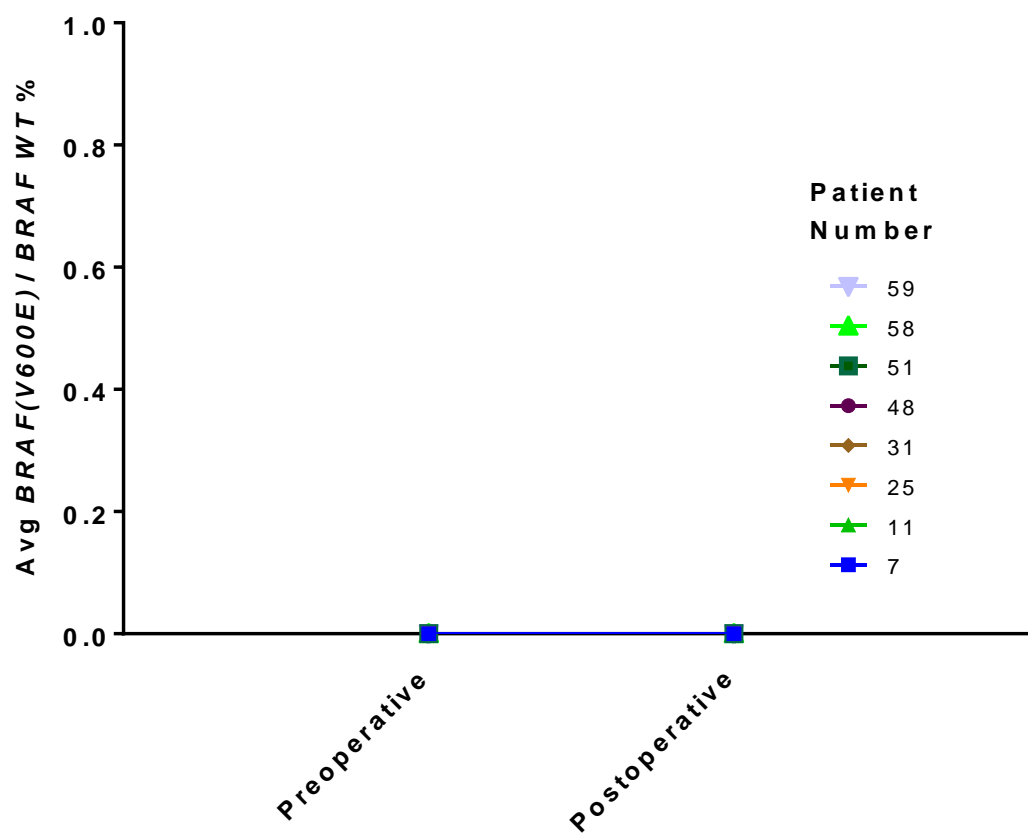


**Figure 12:** *BRAF(V600E)* ctDNA detection as determined by relative amount of *BRAF(V600E)* in patients with paired preoperative and postoperative samples (N = 37). 6/37 (16.2%) patients had detectable levels preoperatively and they all declined postoperatively. Y-axis represents average *BRAF(V600E)* / *BRAF* wildtype percentage. X-axis represents preoperative and postoperative samples. Each datapoint and corresponding line represents an individual patient.



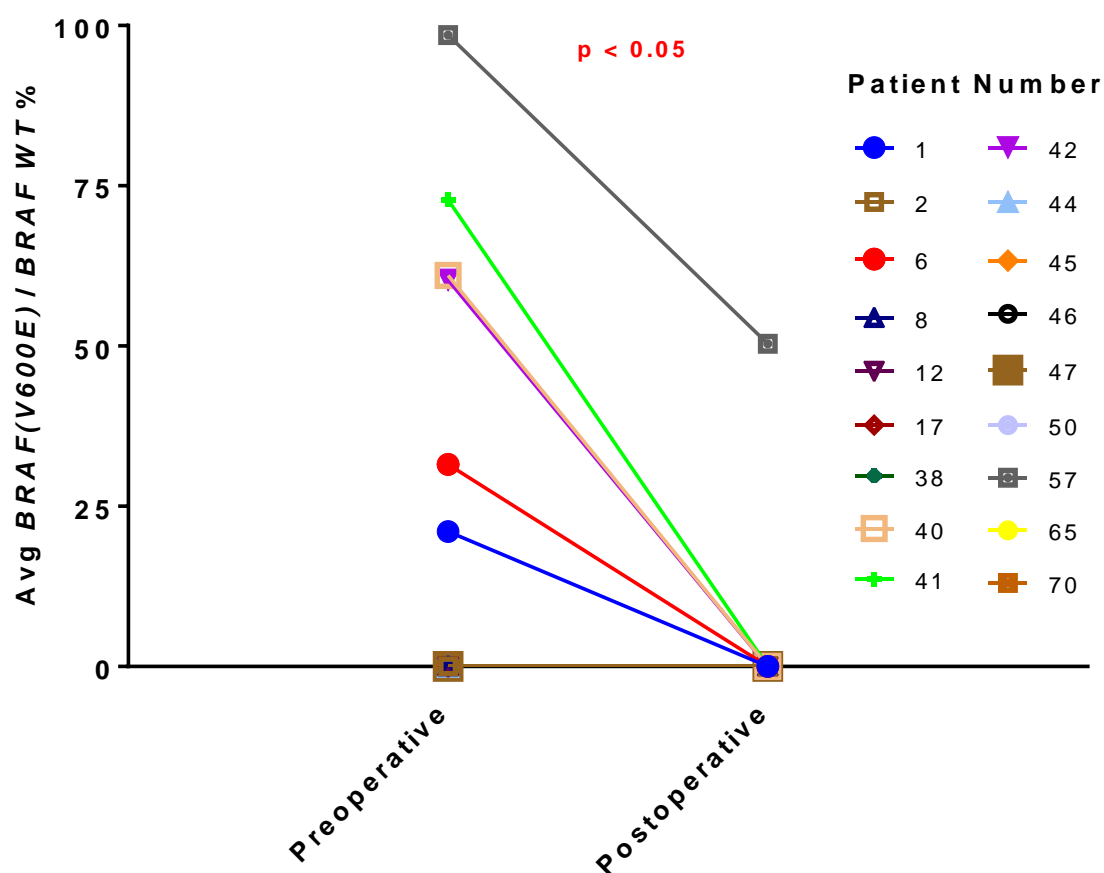
Note: There is overlap of samples 40 and 42 in the graphic representation. Only means have been shown for each sample. Patients with non-detectable ctDNA in either preoperative or postoperative samples are not represented in this figure.

**Figure 13:** *BRAF(V600E)* ctDNA detection as determined by relative amount of *BRAF(V600E)* in paired preoperative and postoperative samples of patients with final pathology suggestive of nodular hyperplasia (N = 8). 0/8 (0%) patients had detectable levels preoperatively and they all declined postoperatively. Y-axis represents average *BRAF(V600E)* / *BRAF* wildtype percentage. X-axis represents preoperative and postoperative samples. Each datapoint and corresponding line represents an individual patient.



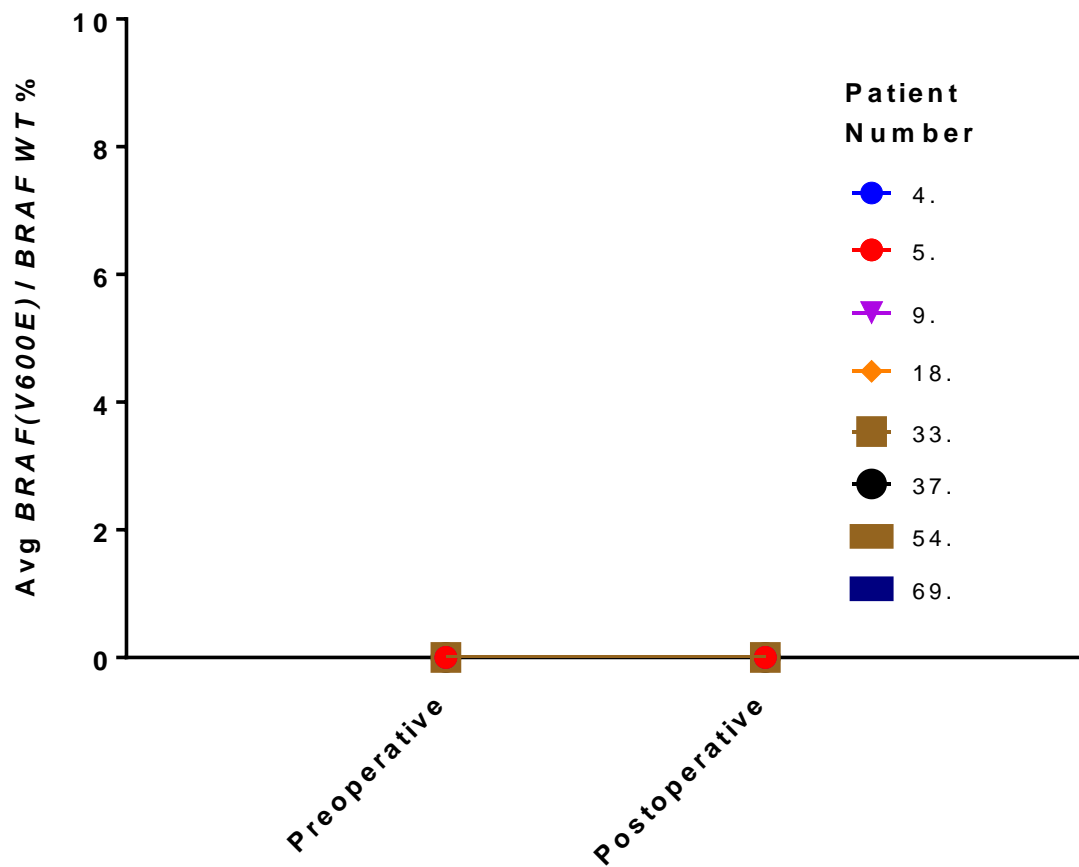
Note: Only means have been shown for each sample.

**Figure 14:** *BRAF(V600E)* ctDNA detection as determined by relative amount of *BRAF(V600E)* in paired preoperative and postoperative samples of patients with final pathology suggestive of classical PTC (N = 17). 6/18 (33.3%) patients had detectable levels preoperatively and they all declined postoperatively. Y-axis represents average *BRAF(V600E)* / *BRAF* wildtype percentage. X-axis represents preoperative and postoperative samples. Each datapoint and corresponding line represents an individual patient.



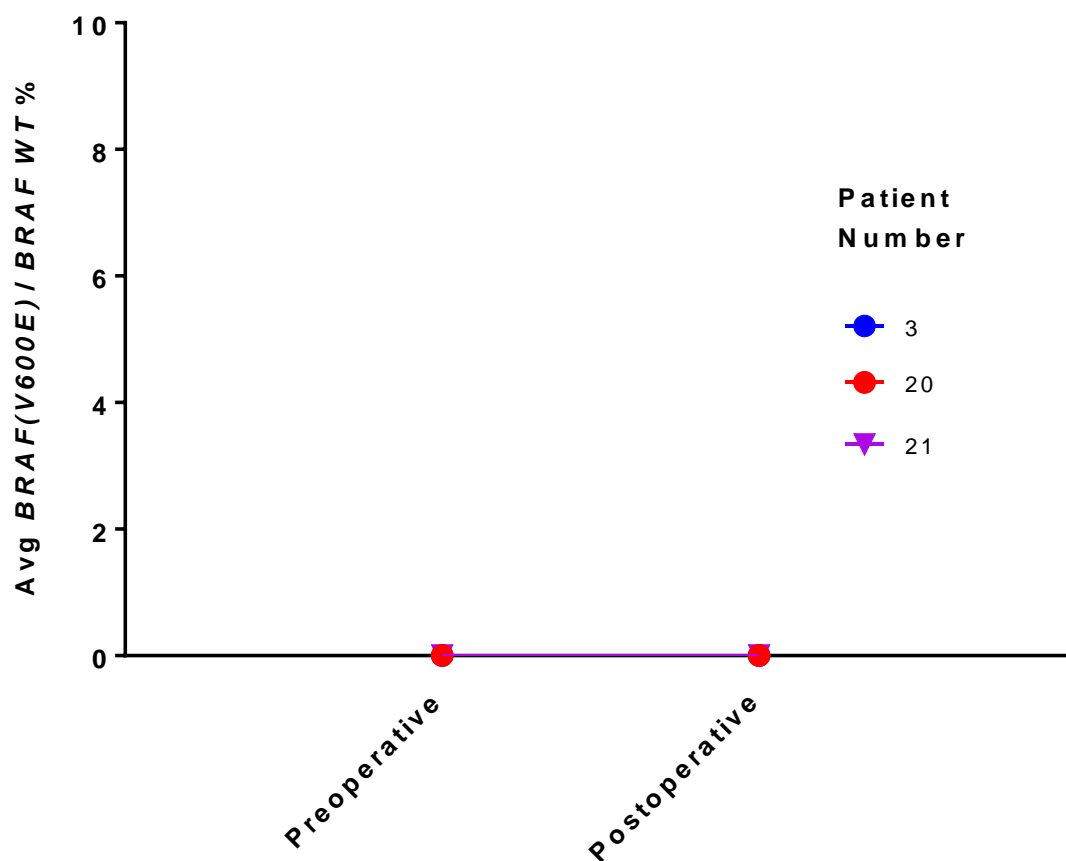
Note: There is overlap of samples 40 and 42 in the graphic representation. Only means have been shown for each sample.

**Figure 15:** *BRAF(V600E)* ctDNA detection as determined by relative amount of *BRAF(V600E)* in paired preoperative and postoperative samples of patients with final pathology suggestive of non-classical PTC (N = 8). 0/8 (0%) patients had detectable levels preoperatively and they remained undetectable postoperatively. Y-axis represents average *BRAF(V600E)* / *BRAF* wildtype percentage. X-axis represents preoperative and postoperative samples. Each datapoint and corresponding line represents an individual patient.



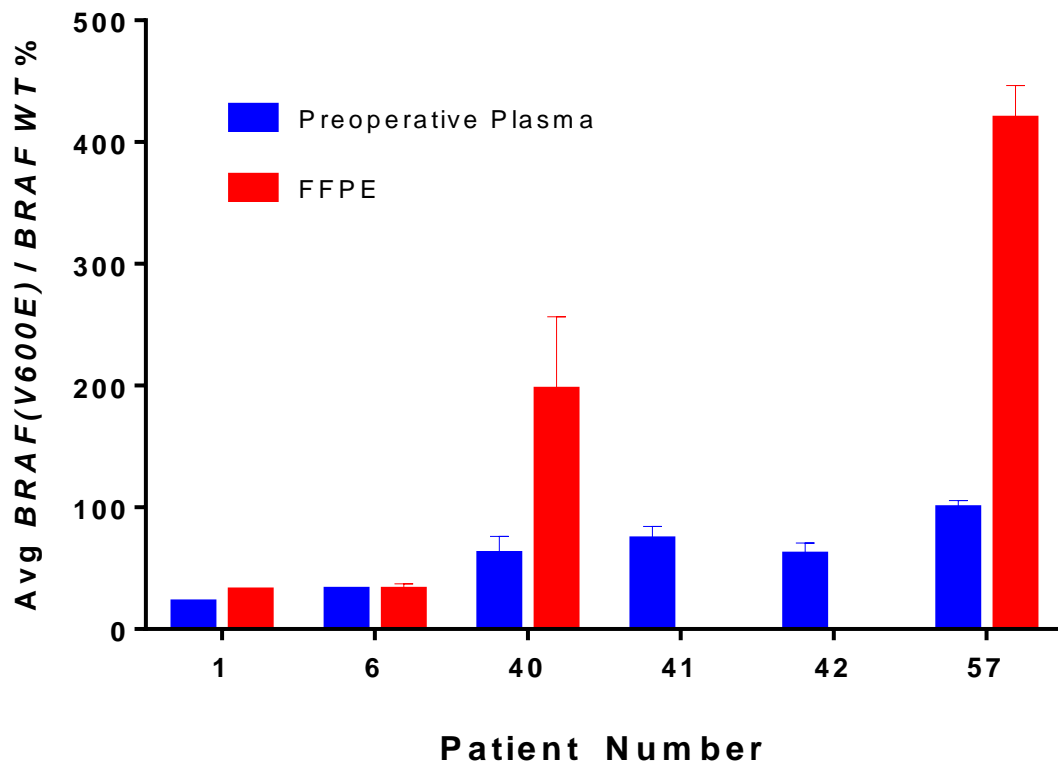
Note: Only means have been shown for each sample.

**Figure 16:** *BRAF(V600E)* ctDNA detection as determined by relative amount of *BRAF(V600E)* in paired preoperative and postoperative samples of patients with final pathology suggestive of FTC (N = 3). 0/3 (0%) patients had detectable levels preoperatively and they remained undetectable postoperatively. Y-axis represents average *BRAF(V600E)* / *BRAF* wildtype percentage. X-axis represents preoperative and postoperative samples. Each datapoint and corresponding line represents an individual patient.



Note: Only means have been shown for each sample.

**Figure 17:** Concordance between FFPE samples and *BRAF(V600E)* ctDNA as determined by relative amount of *BRAF(V600E)* (mean  $\pm$ SE) in paired preoperative and postoperative samples of patients with final pathology suggestive of classical PTC. 4/6 (66.6%) patients had detectable *BRAF(V600E)* ctDNA and corresponding *BRAF(V600E)* in the FFPE samples. Y-axis represents average *BRAF(V600E)* / *BRAF* wildtype percentage. X-axis represents each individual patient.





## Appendix B: Tables

**Table 1:** Clinical features of different types of thyroid cancer.

<b>Tumour type</b>	<b>Prevalence</b>	<b>Sex ratio (female:male)</b>	<b>Age (years)</b>	<b>Lymph-node metastasis</b>	<b>Distant metastasis</b>	<b>Survival rate (5 year)</b>
Papillary thyroid carcinoma	85–90%	2:1–4: 1	20–50	<50%	5–7%	>90%
Follicular thyroid carcinoma	<10%	2:1–3: 1	40–60	<5%	20%	>90%
Poorly differentiated thyroid carcinoma	rare–7%	0.4:1–2.1: 1	50–60	30–80%	30–80%	50%
Undifferentiated thyroid carcinoma	2%	1.5: 1	60–80	40%	20–50%	1–17%
Medullary thyroid carcinoma	3%	1:1–1.2: 1	30–60	50%	15%	80%

Modified from Kondo, et al. <sup>15</sup>

**Table 2:** FNAB results based on Bethesda Thyroid Nodule System

<b>Risk Category</b>	<b>Bethesda</b>	<b>Predicted Risk of Malignancy (%)<sup>a</sup></b>	<b>Malignancy Rate in Literature (%)<sup>b</sup></b>	<b>LHSC Malignancy Rate (%)<sup>c</sup></b>	<b>Recommendations</b>
	Nondiagnostic/Unsatisfactory	1 – 4	16.8	13	Repeat U/S guided FNAB
<b>Low</b>	Benign	0 – 3	3.7	3	Follow
<b>Indeterminate</b>	FLUS/AUS	5 – 15	15.9	13 – 50	Repeat FNAB/Close followup
<b>Intermediate</b>	FN/SFN	15 – 30	26.1	20 – 40	Close followup or diagnostic hemi-thyroidectomy
<b>High</b>	Suspicious for malignancy	60 – 75	75.2	80 – 98	Total thyroidectomy
	Malignant	97 – 99	98.6		

a – <sup>32</sup>, b - data primarily from <sup>32,90-94</sup>, c – unpublished

LHSC – London Health Sciences Centre, FLUS – Follicular Lesion of Unknown Significance, AUS – Atypia of Unknown Significance, FN – Follicular Neoplasm, SFN – Suspicious for Follicular Neoplasm (or Hurthle Cell Neoplasm)

**Table 3:** Genetic alterations in different types of thyroid cancers.

<b>Genetic alteration</b>	<b>Well-differentiated thyroid carcinoma</b>		<b>Poorly differentiated thyroid carcinoma</b>	<b>Undifferentiated thyroid carcinoma</b>
	<b>PTC</b>	<b>FTC</b>		
<b>RET rearrangement</b>	13–43%	0%	0–13%	0%
<b>BRAF mutation</b>	29–69%	0%	0–13%	10–35%
<b>BRAF rearrangement</b>	1%	Unknown	Unknown	Unknown
<b>NTRK1 rearrangement</b>	5–13%	Unknown	Unknown	Unknown
<b>RAS mutation</b>	0–21%	40–53%	18–27%	20–60%
<b>PPARG rearrangement</b>	0%	25–63%	0%	0%
<b>CTNNB1 mutation</b>	0%	0%	0–25%	66%
<b>TP53 mutation</b>	0–5%	0–9%	17–38%	67–88%

Modified from Kondo, et al. <sup>15</sup>

**Table 4:** Clinical applications of ctDNA. ctDNA can be used for diagnosis of disease, predict response to treatment and prognostication by identifying early recurrence and determining tumor burden.

	<b>Application</b>
<b>Diagnostic</b>	Early detection
	Monitoring of minimal residual disease
<b>Predictive</b>	Assessment of molecular heterogeneity of overall disease
	Monitoring of tumor dynamics
	Identification of genetic determinants for targeted therapy
	Evaluation of early treatment response
	Assessment of evolution of resistance in real time
<b>Prognostic</b>	Identification of high risk of recurrence
	Correlation with changes in tumor burden

Modified from Heitzer, et al.<sup>95</sup>

**Table 5:** qPCR reaction setup

<b>Component</b>	<b>Volume</b>	<b>Final Concentration</b>
2x QuantiTect Multiplex PCR Master Mix	10 ul	1 x
20x primer-probe mix 1	0.04 ul	0.4 uM forward primer 0.4 uM reverse primer 0.4 uM probe
20x primer-probe mix 2	0.04 ul	0.4 uM forward primer 0.4 uM reverse primer 0.4 uM probe
RNase-free water	9.56 ul	
Template DNA	0.2 ul	
Total reaction volume	20 ul	

**Table 6:** qPCR Primer and Probe Sequences

Gene	Primer/Probe	Sequences
<i>BRAF</i> non-mutated exon	Forward	5'-TAGGTGATTTTGGTCTAGCTACCGA
	Reverse	5'-GGATCCAGACAACTGTTCAAAGT
	Probe	5'-[JOE]GAATCTCGATGGAGTGGGTC
<i>BRAF(V600E)</i>	Forward	5'-GATGCACTCCAACAAAGAGAACAA
	Reverse	5'-GGTATCCATTGATGCAGAGCTAGA
	Probe	5'-[ROX]TCTCTGGGGAACGGAAGTGA

**Table 7:** qPCR Cycling Conditions

Step	Time	Temperature (°C)
PCR initial activation step	15 min	95
Denaturation	1 min	94
Annealing/extension	1 min 30 sec	62
Number of cycles	40x	

**Table 8:** Baseline patient characteristics and final pathology of the index nodule.

<b>Preoperative Samples = 61</b>		
	Males	21 (34.4%)
	Females	40 (65.6%)
<b>Pathology</b>	Benign	13 (21.3%)
	PTC, classical	26 (42.6%)
	PTC, non-classical	13 (21.3%)
	FTC	3 (4.9%)
	Metastatic PTC, classical	2 (3.3%)
	Metastatic PTC, non-classical	4 (6.6%)



**Table 9:** Paired preoperative and postoperative patient characteristics and final pathology of the index nodule.

<b>Paired Samples = 37</b>		
	Males	12 (32.4%)
	Females	25 (67.6%)
<b>Pathology</b>	Benign	8 (21.6%)
	PTC, classical	18 (48.6%)
	PTC, non-classical	8 (21.6%)
	FTC	3 (8.1%)

**Table 10:** Detection of *BRAF(V600E)* in benign and classical PTC in preoperative plasma ctDNA samples.

	<b>Benign</b>	<b>Classical PTC</b>	<b>p-value<sup>a</sup></b>
<b><i>BRAF(V600E)</i> ctDNA positive</b>	0	8	p < 0.05
<b><i>BRAF(V600E)</i> ctDNA negative</b>	13	18	

a - Fischer's exact test was used to assess for statistical significance.

**Table 11:** Correlation of *BRAF(V600E)* ctDNA and pathologic T-stage, N-stage and ETE in patients with classical PTC patients.

		ctDNA positive	ctDNA negative	p-value <sup>a</sup>
<b>T-stage<sup>b</sup></b>	Low T-stage	1	7	p > 0.05
	High T-stage	5	5	
<b>N-stage</b>	0	1	6	p > 0.05
	1a	3	2	
	1b	2	4	
<b>ETE</b>	Absent	2	7	p > 0.05
	Present	4	5	

a - Fischer's exact test was used to assess for statistical significance.

b – Low T-stage was defined to be T1 and T2. High T-stage was defined to be T3 and T4.

**Table 12:** Concordance between the mutational status of *BRAF(V600E)* in the FFPE index thyroid nodule and preoperative *BRAF(V600E)* ctDNA based on histology.

		<b>FFPE positive</b>	<b>FFPE negative</b>
<b>Nodular Hyperplasia</b>	<b>ctDNA positive</b>	0	0
	<b>ctDNA negative</b>	0	8
<b>PTC, classical</b>	<b>ctDNA positive</b>	4	2
	<b>ctDNA negative</b>	9	3

**Table 13:** Concordance between the mutational status of *BRAF(V600E)* in the FFPE index thyroid nodule and ctDNA. Numbers in each cell represent the total of benign and classical PTC samples.

	FFPE positive	FFPE negative
ctDNA positive	4	2
ctDNA negative	9	11

**Table 14:** Comparison of findings in various studies published on ctDNA in thyroid cancer.

Study	Overall Detection (%) <sup>†</sup>	PTC Detection (%)	Benign Nodule Detection (%)	True Positive (%) <sup>‡</sup>	False Positive (%) <sup>‡</sup>	Comments
Our Study	9/61 (14.8%)	8/26 (29.4%)	0/13 (11.1%)	4/13 (30.8%)	2/11 (18.2%)	Can differentiate between benign and classical PTCs
Chuang et al. <sup>79</sup>	5/29 (17.2%)	5/14 (35.7%)	0/9 (0%)	3/5 (60%)	0/9 (0%)	
Cradic et al. <sup>78</sup>	20/193 (10.4%)	20/173 (11.6%)	n/a	8/42 (19%)	1/14 (7.1%)	Only included thyroid cancer patients - true positive and false positive rates do not account for nodular hyperplasia patients
Kim et al. <sup>80</sup>	3/77 (3.9%)	3/72 (4.2%)	0/5 (0%)	3/49 (6.1%)	0/23 (0%)	Only included thyroid cancer patients - true positive and false positive rates do not account for nodular hyperplasia patients
Pupilli et al. <sup>77</sup>	n/a	14/22 (63.6%)	n/a	11/13 (84.6%)	3/9 (33.3%)	Used Receiver Operative Curve to determine the sensitivity and specificity. In Thy 3 group false positive rate was 50% based on the cut off values.
Zane et al. <sup>81</sup>	0/181 (0%)	n/a	n/a	0/68 (0%)	n/a	Unable to analyze ctDNA

<sup>†</sup> – Overall detection in plasma

<sup>‡</sup> – Concordance between *BRAF(V600E)* ctDNA and index thyroid nodule irrespective of the final pathology. FFPE tissue samples were used as standards to calculate true positives and false negative rates. True positive = true positive on FFPE and ctDNA / Total number of FFPE positive samples. False positive = ctDNA positive but FFPE negative/Total number of FFPE negative samples.

## Appendix C: TNM Classification System for Differentiated Thyroid Carcinoma

Definition		
T1	Tumor diameter 2 cm or smaller	
T2	Primary tumor diameter >2 to 4cm	
T3	Primary tumor diameter >4 cm limited to the thyroid or with minimal extrathyroidal extension	
T4 <sub>a</sub>	Tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve	
T <sub>b</sub>	Tumor invades prevertebral fascia or encases carotid artery or mediastinal vessels	
T <sub>x</sub>	Primary tumor size unknown, but without extrathyroidal invasion	
N0	No metastatic nodes	
N1 <sub>a</sub>	Metastases to level VI (pretracheal, paratracheal, and prelaryngeal=Delphian lymph nodes)	
N1 <sub>b</sub>	Metastasis to unilateral, bilateral, contralateral cervical or superior mediastinal nodes	
N <sub>x</sub>	Nodes not assessed at surgery	
M0	No distant metastases	
M1	Distant metastases	
M <sub>x</sub>	Distant metastases not assessed	
Stages		
	Patient age < 45 years	Patient age 45 years or older
Stage 1	Any T, any N, M0	T1, N0, M0
Stage II	Any T, any N, M1	T2, N0, M0
Stage III		T3, N0, M0
		T1, N1 <sub>a</sub> , M0
		T2, N1 <sub>a</sub> , M0
		T3, N1 <sub>a</sub> , M0
Stage IVa		T4 <sub>a</sub> , N0, M0
		T4 <sub>a</sub> , N1 <sub>a</sub> , M0
		T1, N1 <sub>b</sub> , M0
		T2, N1 <sub>b</sub> , M0
		T3, N1 <sub>b</sub> , M0
		T4 <sub>a</sub> , N1 <sub>b</sub> , M0
Stage IVb		T4 <sub>b</sub> , Any N, M0
Stage IVc		Any T, Any N, M1

Modified from Edge and American Joint Committee on Cancer.<sup>96</sup>

## Appendix D: Research Ethics Board Application

### General Info

FileNo: 103985

Title: Detection of Circulating Tumor DNA in Thyroid Cancer

Start Date: 10/02/2014

End Date: 31/08/2018

Keywords: thyroid cancer,biomarkers,circulating tumor DNA,screening test

### Project Members

#### Principal Investigator

Prefix: Dr.

Last Name: Nichols

First Name: Anthony

Affiliation: Schulich School of Medicine and Dentistry\Otolaryngology

Rank: Assistant Professor

Gender: Male

Email:

Phone1:

Phone2:

Fax:

Mailing Address:

Institution:

Country:

Comments:

#### Others

Rank	Last Name	First Name	Affiliation	Role In Project
	Rachinsky	Irina	Schulich School of Medicine and Dentistry\Imaging	Co-Investigator
Lecturer	Macneil	Danielle	Schulich School of Medicine and Dentistry\Otolaryngology	Co-Investigator
	Van Uum	Stan	Schulich School of Medicine and Dentistry\Medicine-Dept of	Co-Investigator
	Fung	Kevin	Schulich School of Medicine and Dentistry\Oncology	Co-Investigator
	Barrett	John		Co-Investigator
Adjunct Professor	Theurer	Julie	Health Sciences\Communication Sciences & Disorders	Co-Investigator



Associate Professor	Yoo	John	Schulich School of Medicine and Dentistry\Otolaryngology	Co-Investigator
Associate Professor	Kwan	Keith	Schulich School of Medicine and Dentistry\Pathology	Co-Investigator
Research Assistant	Partridge	Allison	Schulich School of Medicine and Dentistry\Medicine-Dept of	Research Support Staff
Resident	Patel	Krupal	Schulich School of Medicine and Dentistry\Otolaryngology	Student

## Common Questions

### 1. Registration Information

#	Question	Answer
1.1	Do you confirm that you have read the above information and that based on that information you are completing the correct form?	Yes
1.2	Has this study been submitted to any other REB? If yes, please include the approval letter (or relevant correspondence).	No
1.3	If YES is selected in question 1.2 above, please indicate where this project has been submitted and when.	
1.4	Indicate the funding source for this study or if there is no funding simply indicate "None".	Self-funded
1.5	If you have indicated a funding source in question 1.4 above, please specify the name of the funding source selected as well as the title of the grant and if applicable the ROLA number.	Translational Head and Neck Cancer Research Fund (philanthropic funds)
1.6	Is this a sequel to previously approved research?	No
1.7	If YES is selected in question 1.6 above, what is the REB number and what are the differences?	
1.8	Is this a student project?	Yes - Resident/Fellow
1.9	Is this a multi-site study?	No
1.10	If YES has been selected in question 1.9 above, name the lead site and project leader for the study. If the study	

	is administered by a Coordinating or Contract Research Organization (CRO) provide the name and contact information.	
1.11	Please list the names of ALL Local (Western affiliated) team members who are working on this project. Please ALSO list their ROLE in the project, i.e. what exactly is it that the team member will do in this study? Please see the “i” for this question for instructions on how to link their Romeo accounts to this form so they have access to it.	Head and Neck Surgeons (involved in patient recruitment, consent, coordinating blood draws) -John Yoo, Kevin Fung, Anthony Nichols, Danielle MacNeil, Krupal Patel Pathologists (responsible for reviewing the thyroid pathology and obtaining punch biopsies from focuses of tumor) -Bret Wehrli and Keith Kwan Post-Doctoral Fellow (responsible for study coordination, data interpretation). -Julie Theurer Scientists (responsible for study design, mutational detection, data analysis, manuscript preparation) -John Barrett, Anthony Nichols, Krupal Patel
1.12	Are the investigator(s) based at any of the sites below or will the study utilize any patient data, staff resources or facilities within any of these sites? (Please indicate all applicable sites and read the associated notes found in the blue information icon above)	LHSC - Victoria Hospital
1.13	If this form was started by a team member, has the role of Principal Investigator been changed to the Faculty member who will hold this role for the study? This is required for review of your submission, and any forms submitted without this change being made will be returned without being reviewed. (The blue information “i” has the instructions on how to change the role of PI.)	Not Applicable – Principal Investigator is the one who started the submission form
1.14	Please provide a lay summary of the study (typically fewer than 5 lines).	Thyroid nodules are very common, and the majority are benign. Nodules are typically evaluated with a needle biopsy, however a large proportion of the needle biopsies are non-diagnostic and surgery is offered. Needle biopsies can also be very uncomfortable for patients. A non-invasive blood test that can differentiate between benign and malignant thyroid nodules would be of tremendous value to better

		guide the care of these patients. DNA that is shed by the tumor into the blood stream can be detected and serve as a screening test.
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## 2. Methodology

#	Question	Answer
2.1	Outline the study rationale including relevant background information and justification. Cite references where appropriate.	<p>Thyroid nodules are extremely common, and can be indentified in over 50% of the population on ultrasounds. The first line of investigation for these nodules is a fine needle aspiration (FNA) biopsy, however a significant fraction of FNA results are indeterminate. These patients typically undergo a hemithyroidectomy, however only 15% of indeterminate biopsies prove to be cancer. In addition, needle biopsies can be very uncomfortable for patients, particularly if multiple passes are needed. Ideally a accurate non-invasive blood test that can distinguish between benign and malignant nodules would be of great utility. Such a test would both avoid unnecessary surgery in patients with benign disease and guide surgeons towards total thyroidectomy in patients with malignancy. Another challenge in thyroid disease is the follow up of thyroid cancer. Thyroglobulin is secreted specifically from thyroid tissue. After thyroidectomy, rising thyroglobulin levels are useful to detect recurrence in the majority of cases. However, small remenants of thyroid tissue are let in situ after thyroidectomy that result in an elevated thyroglobulin level despite the absence of residual cancer. More troublesome is a small cohort of thyroid cancer patients that become thyroglobulin negative despite demonstrable recurrent disease. Improved diagnostics would be helpful to monitor this cohort. It has been well established that cancers shed DNA that can be detected in the bloodstream. Mutations in oncogenes and tumor suppressors that are found in cancers should not be found in the body of healthy patients.</p>

		<p>Studies in other cancers have shown that the detection of circulating tumor DNA (ctDNA) in cancer patients can serve as a ultra-sensitive test of disease status. Unlike the majority of malignancies, the majority of well differentiated thyroid cancers (~80%) have either a specific fusion (RET/PTC or PAX8/PPARgamma) or hotspot mutations in well described oncogenes (RAS, PIK3CA, BRAF). Thus a rather small panel of mutations can be tested for in plasma samples as a screening and surveillance test for a large fraction of thyroid cancers. Although well described in other cancers, ctDNA has largely not been investigated in thyroid disease with the exception of a pilot study that evaluated only BRAF mutations (PUBMED ID: 19850689).</p>
2.2	Please provide a clear statement of the purpose and objectives of this project (one page maximum).	<p>1) To determine the ability to detect BRAF, PIK3CA, RAS mutations and RET/PTC and PAX9/PPARgamma fusions in the plasma of patients with thyroid nodules using a real time PCR assay. 2) To determine the reliability of ctDNA detection with malignancy on final pathology.</p>
2.3	Describe the study design/methodology and attach all supporting documents in the attachments tab.	<p>Patients undergoing thyroid surgery with benign, indeterminate and malignant needle biopsies will be identified for study. Ten mL of blood will be drawn at the time of routine bloodwork preoperatively and at one month, three month, six month and one year follow-up with the surgeon after surgery. An additional 10 ml of blood sample will be collected immediately after surgery once the specimen has been removed. The blood will be centrifuged and the plasma removed. DNA will be extracted from the plasma and tested for mutations in the previously mentioned mutations and fusions by real-time polymerase reaction (PCR). The cycle threshold difference between the wild type and mutation specific primers will be used to determine positivity. Positive PCR assays pre and post-operatively will be compared to the results of surgery. A subset of the thyroidectomy</p>

		samples (100) will be screened by a pathologist. Area of tumor will be identified and multiple 1mm cores (up to 5) will be taken from the center of the tumor and DNA will be extracted. The above mentioned mutations and fusions will be tested for by real-time PCR.
2.4	Indicate the inclusion criteria for participant recruitment.	1) Age 18 or older 2) Scheduled to undergo partial or total thyroidectomy for nodules
2.5	Considering your inclusion criteria listed above, what is the basis to exclude a potential participant?	1) Previous cancer that is known to be positive for one of the tested mutations (i.e. melanoma which may contain BRAF mutations).
2.6	If using patients, describe the usual standard of care at the study site(s) for this population (including diagnostic testing, frequency of follow up visits).	All thyroidectomy patients undergo pre and post-operative testing to assess thyroid function (TSH, T3, T4), parathyroid levels (PTH), and calcium levels. At the very least this occurs at initial consultation and at the follow up at ~3-4 weeks to discuss the pathology. Thus the patient will be undergoing a blood draw at these appointments. The sample (10mL) for mutational testing would be draw at the same time using blood tubes provided by the principal investigator, thus no additional blood draws or hospital resources are required for this study.
2.7	Describe the study procedures and any study specific testing that will be done, outside of standard care.	Blood and tumor mutational analysis by real-time PCR will be tested. As this is not done in a certified lab setting and the significance of the findings are not yet known, results will not be reported to the patient.
2.8	How many participants over the age of 18 from London will be enrolled in your study? This includes hospital and university sites within London.	Up to 400.
2.9	How many participants under the age of 18 from London will be enrolled in your study? This includes hospital and university sites within London.	0
2.10	How many participants over the age of 18 will be included at all study locations? (London + Other locations = Total)	Up to 400.

2.11	How many participants under the age of 18 will be included at all study locations? (London + Other locations = Total)	0
2.12	Describe the method(s) of data analysis.	Mutational and fusion protein analysis will be carried out by real-time PCR testing. Pre and post-operative mutational levels will be compared as will the presence of mutations in patients with benign and malignant thyroid nodules.
2.13	How will the results of this study be made public?	Peer reviewed publication
2.14	If report to participants or other is selected above, please explain.	
2.15	Briefly provide any plans for provision of feedback of results to the participants.	No feedback will be provided as the significance of the mutation analysis is not yet determined and the testing is not carried out in a certified lab.
2.16	Does this study include any use of deliberate deception or withholding of key information that may influence a participant's performance or response?	No
2.17	If YES in question 2.16 above, describe this process and justification including how the participants will be debriefed at some point. Please include the debriefing script.	

### 3. Risks and Benefits

#	Question	Answer
3.1	List any potential anticipated benefit to the participants.	None other than potentially improving the care of future patients with thyroid disease.
3.2	List the potential benefits to society.	Blood mutational testing may offer a non-invasive way to assess the risk of malignancy in thyroid nodules. This may help to avoid unnecessary surgery in patients with benign nodules and identify patients that would benefit from total thyroidectomy versus two step thyroidectomy. This study may also lead to a more accurate way to follow patients with thyroid cancer.
3.3	List any potential risks to study participants.	None.

3.4	List any potential inconveniences to daily activities.	None. All the additional blood samples would be collected at the time of blood draws needed for clinical care.
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#### 4. Recruitment and Informed Consent

#	Question	Answer
4.1	How will potential participants be contacted and recruited? Select all that apply. A copy of all recruitment tools that will be used must be included with this submission in the attachments tab.	Investigators will approach their own patients/students
4.2	Please explain in detail your selection from 4.1 and how it will be used to recruit participants.	The investigators including the four surgeons (Yoo, Nichols, Fung, MacNeil, Patel (resident)) and three endocrinologists (Morrison, Van Uum, Paul) will be responsible for patient recruitment in clinic preoperatively. The letter of information and consent will be used to provide a description of the study to potential subjects. Drs. Nichols, Barrett and Mymryk will be responsible for the mutational testing.
4.3	Which research team members will be recruiting the potential participants?	See above.
4.4	Does the Principal Investigator have any relationship to the potential participants?	Yes, the principal investigator will be recruiting subjects, some of which will be his own patients.
4.5	Does the person recruiting the participants have any relationship or hold any authority over the potential participants?	Yes
4.6	If you have answered "Yes" to either 4.4 or 4.5, please explain here.	The majority of time, the person recruiting participants will be the treating physician. As this is a low/no risk study the chance of harm is negligible.
4.7	What method of obtaining consent will you use for participants? A copy of all forms being used for obtaining consent must be included with this submission.	Written Consent
4.8	If you are unable to obtain consent or assent using one of the methods listed above, please explain here.	

4.9	Indicate if you will be recruiting from any of the following groups specifically for this study. (select all that apply)	Patients
4.10	Will minors or persons not able to consent for themselves be included in the study?	No
4.11	If YES is selected in question 4.10 above, describe the consent process and indicate who will be asked to consent on their behalf and discuss what safeguards will be employed to ensure the rights of the research participant are protected.	
4.12	When the inability to provide an informed consent is expected to be temporary, describe what procedures will be used to regularly assess capacity and to obtain consent if the individual later becomes capable of providing consent. Alternatively, if diminished capacity is anticipated for the study population, describe the procedure used to assess capacity and obtain ongoing consent.	Only patients able to consent at the time of preoperative consultation will be recruited.
4.13	List any anticipated communication difficulties:	None
4.14	Describe the procedures to address any communication difficulties (if applicable):	not applicable.
4.15	Indicate what compensation, if any, will be provided to subjects. For example, reimbursement for expenses incurred as a result of research, description of gifts for participation, draws and/or compensation for time. Include a justification for this compensation.	none.

### 5. Confidentiality and Data Security

#	Question	Answer
5.1	Are you collecting personal identifiers for this study?	Yes
5.2	Identify any personal identifiers collected for this study.	Full name Health card number Date of birth



5.3	If you checked any of the personal information in 5.2 above, please explain and justify the collection of this identifier.	Patients will need to be followed to determine if there is a difference in pre and post operative mutational status and if serum mutational status correlates with the tumor. This will require creation of a password protected secure database located on a hospital network drive (S drive) behind the hospital firewall.
5.4	Where will information collected as part of this study be stored? (select all that apply)	University or Hospital network drive (specify below)
5.5	If you have indicated any of the locations in question 5.4, please specify here.	We will create a password protected secure database stored on a hospital drive behind the hospital firewall.
5.6	If identifiable participant information is stored on a hard drive or portable device, the device must be encrypted. Describe encryption being used.	Not applicable.
5.7	How will you record study data?	Data Collection Form Other
5.8	If you select "Other" in 5.7, please explain why here:	Patient data will be immediately entered into the secure database on the network server once an individual is recruited.
5.9	Describe the coding system to protect identifiable information or explain why the data must remain identifiable.	All identifying information will only be stored in the secure database. Collected samples will be labelled with participant number only - i.e. #1, #2, #3, #4 and so on, to maintain patient confidentiality/privacy.
5.10	How will you store and protect the master list, signed original letters of information and consent documents or other data with identifiers?	Paper file (Required Protection: Locked cabinet in locked institutional office) Electronic file (local) (Required Protection: Password protected computer on a secure network behind institutional firewalls - specify location)
5.11	If any options are selected above, please provide the specific details here.	Password protected secure database stored on a hospital drive behind the hospital firewall.
5.12	How will you store and protect data without identifiers?	Not applicable.
5.13	If you plan to de-identify the study data, please describe the method of de-identification.	Not applicable.
5.14	How long will you keep the study data?	For 5 years after the completion of the study.

5.15	How will you destroy the study data after this period? (If applicable)	Delete the database.
5.16	Does this study require you to send data outside of the institution where it is collected? This includes data taken off-site for analysis. Please note that Western/Robarts are considered off-site locations for hospital/Lawson based studies, and vice-versa.	No
5.17	Where will the data be sent?	Not applicable.
5.18	Does the data to be transferred include personal identifiers? If yes, a data transfer agreement may be necessary.	No
5.19	List the personal identifiers that will be included with the data sent off-site.	Not applicable.
5.20	If you have answered yes to 5.18 please indicate how the data will be transmitted	
5.21	Please specify any additional details on data transmission below.	
5.22	Will you link the locally collected data with any other data sets?	No
5.23	If YES is selected in question 5.22 above, identify the dataset	
5.24	If YES is selected in question 5.22 above, explain how the linkage will occur.	
5.25	If YES is selected in question 5.22 above, provide a list of data items contained in the dataset.	
5.26	Will the data be entered into a database for future use?	Yes
5.27	If YES is selected in question 5.25 above, please specify where it will be stored, who the custodian will be, who will have access to the database and what security measures will be in place.	The data will be stored on a password protected database on a hospital server (S Drive) behind the hospital firewall. Only the study investigators will have access to the information.
5.28	Please list agencies/groups/persons outside of your local research team who will have access to the identifiable data and indicate why access is required.	Not applicable.
5.29	Western University policy requires that that you keep data for a minimum of 5 years. Please indicate if you are	Yes, data will be kept for 5 years after completion of the study.

	keeping data in accordance to this policy, otherwise please comment on how your data retention will differ from University policy and why. If you will be archiving the data, please explain why and how here.	
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#### 6. Conflict of Interest

#	Question	Answer
6.1	Will any investigators, members of the research teams, and/or their partners or immediate family members function as advisors, employees, officers, directors or consultants for this study?	No
6.2	Will any investigators, members of the research team, and/or their partners or immediate family members have a direct or indirect financial interest (including patents or stocks) in the drug, device or technology employed in this research study?	Yes
6.3	Will any investigators, members of the research team, and/or their partners or immediate family members receive any personal benefit (apart from fees for service) as a result of, or connects to this study?	Yes
6.4	If YES is selected in any of the above, please describe the nature of the conflict of interest and how all conflict(s) of interest will be managed.	The results of this study may potentially lead to patents and a commercial product. We will take the appropriate steps within the Western University policy if we wish to commercialize the findings.

#### 7. Industry Sponsored Protocols

#	Question	Answer
7.1	Is this an industry sponsored protocol?	No
7.2	Billing Information - Company Institution:	
7.3	Contact Person:	
7.4	Email of Contact Person:	
7.5	Street Address:	
7.6	City:	

7.7	Country:	
7.8	Province/State:	
7.9	Phone Number:	
7.10	Fax:	
7.11	Contract and/or protocol reference number required:	
7.12	Additional Sponsor Reference or contact information:	
7.13	Do you wish to apply for a REB Administration Fee Adjustment/Waiver?	
7.14	If YES to question 7.13 above, provide a brief written explanation indicating how the funding will be used, who will own the data or any intellectual property arising from the agreement and indicate if there are any restrictions imposed upon the investigator by the sponsor and, if so, what they are.	
7.15	Do you agree to the Conditions for Industry Funded Research Investigators?	
7.16	Do you agree to provide supporting documents? (These can be added in the attachments section)	

### 8. Confirmation of Responsibility

#	Question	Answer
8.1	As the Principal Investigator I have read the Tri-Council Policy Statement 2 and Western University's Guidelines on Research Involving Human Subjects and agree to abide by the guidelines therein: <a href="http://uwo.ca/research/ethics/health_sciences/d_guidelines.html">http://uwo.ca/research/ethics/health_sciences/d_guidelines.html</a>	Yes
8.2	I attest that all Collaborators working on this Research Study (co-investigators, students, post-docs, etc.) have reviewed the protocol contents and are in agreement with the protocol as submitted;	Yes
8.3	All Collaborators have read the Tri-Council Policy Statement 2 and Western University's Guidelines on Research Involving Human Subjects and agree to abide by the guidelines therein;	Yes
8.4	The Collaborators and I will adhere to the Protocol and Letter(s) of Information as approved by the REB;	Yes

8.5	Should I encounter any changes or adverse events/experiences, I will notify the REB in a timely manner.	Yes
8.6	If the Research Study is funded by an external sponsor, I will not begin the Research Study until the contract/agreement has been approved by the appropriate university, hospital, or research institute official.	Yes
8.7	Have you exported a copy of this submission to Word using the "Export to Word" button? Note that you will be unable to submit future revisions if this is not done.	Yes
8.8	Have you uploaded the following documents, if applicable, to the attachments tab? Incomplete submissions will be returned without being reviewed.	Letter(s) of Information and Consent Documentation

### 9. Confirmation of Responsibility - Student

#	Question	Answer
9.1	Is this a student project?	Yes
9.2	As the Student I have read the Tri-Council Policy Statement 2 and Western University's Guidelines on Research Involving Human Subjects and agree to abide by the guidelines therein: <a href="http://uwo.ca/research/ethics/health_sciences/d_guidelines.html">http://uwo.ca/research/ethics/health_sciences/d_guidelines.html</a>	Yes
9.3	I will adhere to the Protocol and Letter(s) of Information as approved by the REB;	Yes
9.4	I will notify the Principal Investigator as soon as possible if there are any changes or adverse/experiences, violations/deviations in regards to the Research Study.	Yes

### Attachments

Description	File Name	Version Date
Letter of Information and Consent	Thyroid Circulating Tumor REB v10.docx	22/06/2013
	103985 - Nichols.pdf	22/06/2013
	Thyroid Circulating Tumor REB v20 - with highlights.docx	25/09/2013
	Thyroid Circulating Tumor REB v20.docx	25/09/2013
	Thyroid Circulating Tumor REB v20.pdf	25/09/2013
	Data Collection Form - Detection of Circulating Tumor DNA in Thyroid Cancer.xls	24/09/2013

Thyroid Circulating Tumor Pathology Approval (Received for information only)	Thyroid Circulating Tumor Pathology Approval.PDF	27/08/2013
	103985 - Response letter to the concerns raised by HSREB.pdf	28/08/2013
	103985 - Response letter to the concerns raised by HSREB.doc	28/08/2013
Data Collection Form - Detection of Circulating Tumor DNA in Thyroid Cancer	Data Collection Form - Detection of Circulating Tumor DNA in Thyroid Cancer v20.xls	04/10/2013
	Thyroid Circulating Tumor REB v30.docx	04/10/2013
	Thyroid Circulating Tumor REB v30 - with highlights.docx	04/10/2013
Recommendations	103985 - Nichols.pdf	26/07/2013
With updated LOI including information about disclosure of results	Thyroid Circulating Tumor REB v40 - with highlights.docx	31/10/2013
With updated LOI including information about disclosure of results	Thyroid Circulating Tumor REB v40.docx	31/10/2013
With updated LOI including information about disclosure of results	Thyroid Circulating Tumor REB v40.pdf	31/10/2013
	Nichols 103985.pdf	13/11/2013
	103985 - with highlights.doc	
	103985.doc	
(Received Dec 9/13) Inclusion of 40 metastatic disease patients	103985.pdf	
	Thyroid Circulating Tumor REB v50 - with highlights.docx	
	Thyroid Circulating Tumor REB v50.docx	
	Thyroid Circulating Tumor REB v50.pdf	
	Thyroid Circulating Tumor REB v5.0 - metastatic papillary thyroid cancer.pdf	
	Thyroid Circulating Tumor REB v5.0 - metastatic papillary thyroid cancer.docx	17/09/2013
	Thyroid Circulating Tumor REB v6.0 - metastatic papillary thyroid cancer.pdf	09/02/2014

	DOC021014-02102014154502-0002.pdf	10/02/2014
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## Appendix E: Research Ethics Board Approval



**Western  
Research**

Research Ethics

Use of Human Participants - Initial Ethics Approval Notice

Principal Investigator: Dr. Anthony Nichols  
 File Number: 103865  
 Review Level: Delegated  
 Protocol Title: Detection of Circulating Tumor DNA in Thyroid Cancer  
 Department & Institution: Schulich School of Medicine and Dentistry/Otolaryngology, Western University  
 Sponsor:  
 Ethics Approval Date: November 13, 2013 Expiry Date: August 31, 2018  
 Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Western University Protocol		2013/06/22
Other	Thyroid Circulating Tumor Pathology Approval (Received for information only)	2013/08/27
Other	Data Collection Form - Detection of Circulating Tumor DNA in Thyroid Cancer	2013/10/04
Letter of Information & Consent	With updated LOI including information about disclosure of results	2013/10/31

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced protocol(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00300943.

Signature \_\_\_\_\_

Ethics Officer to Contact for Further Information

<input type="checkbox"/> Erica Basile	<input type="checkbox"/> Grace Kelly	<input type="checkbox"/> Mira Melnikoff	<input checked="" type="checkbox"/> Vicki Tran
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## Research Ethics

## Use of Human Participants - Revision Ethics Approval Notice

Principal Investigator: Dr. Anthony Nichols  
 File Number: 03883  
 Review Level: Delegated  
 Protocol Title: Detection of Circulating Tumor DNA in Thyroid Cancer  
 Department & Institution: Schulich School of Medicine and Dentistry/Otolaryngology, Western University  
 Sponsor:  
 Ethics Approval Date: February 10, 2014 Expiry Date: August 31, 2016  
 Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Revised Western University Protocol	(Received Dec 9/13) Inclusion of 40 metastatic disease patients	
Letter of Information & Consent		2014/02/09

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph C. Iltis. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Signature \_\_\_\_\_

Ethics Officer to Contact for Further Information

Erke Basile	Grace Kelly	Mike McLean	<input checked="" type="checkbox"/> Yikki Chan
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This is an official document. Please retain the original in your files.

# Appendix F: Letter of Information and Informed Consent – Metastatic Thyroid Cancer Patients



Otolaryngology - Head and Neck Surgery

Detection of Circulating Tumor DNA in thyroid nodular disease  
 PI: Dr. Anthony Nichols  
 Letter of Information and Consent for Papillary Thyroid Cancer Patients

Letter of Information and Consent for Papillary Thyroid Cancer Patients

Detection of Circulating Tumor DNA in Thyroid Nodular Disease

Principal Investigator: Dr. A. Nichols

Co-Investigators: Drs. J. Barrett, J. Mymryk, J. Yoo, K. Fung, D. MacNeil, D. Morrison, I. Rachinsky, S. Van Uum, T. Paul.

## Request for Participation:

You are being invited to participate in a research study to develop a blood test to identify and for follow-up of thyroid cancer in thyroid nodules because you have metastatic papillary thyroid cancer. Patients over the age of 18 years and have been diagnosed with thyroid nodules or thyroid cancer or metastatic papillary thyroid cancer are to be included in this study. Patients who are below the age of 18 years or have had previous cancers that are known to be positive for mutations to be detected such as melanoma will be excluded from this study. This letter of information is provided for your review to obtain consent for your participation in the proposed study.

A team of scientists who specialize in thyroid research at the London Regional Cancer Program will analyse DNA in your blood sample to look for changes in DNA due to a potential thyroid cancer. This could lead to important information in the future about how to diagnose, treat and follow-up patients with thyroid nodules. This research requires samples from approximately four hundred patients in order to have enough information to produce meaningful results. Approximately 40 patients with metastatic papillary thyroid cancer will be included as part of the study as well. Patients with metastatic papillary thyroid cancer have been asked to participate in this study because previous studies have shown that papillary thyroid cancers express the genetic mutations we are interested in. Thus these patients would act as positive controls for our methods of detection of the genetic mutations. If you agree to participate, information will be collected about your treatment, your follow-up visits to the clinic, and the extent of the cancer after treatment. Only the study investigators will have current and future access to this information.

## Participation Details:

### Blood Sample

As part of the routine work up for thyroid cancer, you will undergo thyroid blood tests before and approximately one month after your operation. An additional 10 mL blood sample will be taken at the time of routine blood work before and after treatment and will be tested for specific mutations (genetic changes) that are thought to be correlated with thyroid cancer.

### Results of Participation:

You do not need to spend any extra time participating in this study, besides the time needed for your treatment, routine blood work and your follow-up visits with your doctor. There is no risk involved with your participation in this study above the standard blood work.

\_\_\_\_\_ Initials

Version 2.0 September 17, 2013





# Detection of Circulating Tumor DNA in thyroid nodular disease

PI: Dr. Anthony Nichols

## Letter of Information and Consent for Papillary Thyroid Cancer Patients

As mentioned previously, this is a preliminary study investigating the utility of detecting thyroid tumor DNA in a blood test. The detection will be done in a research lab that is not certified for medical purposes. Thus the results of the presence or absence of abnormal circulating thyroid tumor DNA will not be disclosed to you.

We are also asking for your permission to enter your medical information (including your age, tumour size, type of tumor) into a database so that we can keep track of your treatment, and outcome. The results from this research study may lead to valuable information including better treatment for thyroid disease. There may be no direct benefits to you for participating in this study, but the blood and tumor samples and information we collect may help other patients with thyroid disease in the future.

### **Voluntary Participation:**

Participation in this study is voluntary. You may refuse to participate or withdraw from the study at any point with no effect on your future care. If you do withdraw from the study, any information collected up until that point will be kept in the database but no more information will be collected. The data collected must be retained in order to protect the integrity of research up to that point.

### **Confidentiality:**

Every effort will be made to ensure that the confidentiality of your medical information is protected. Your medical information will be recorded in a secure computer database with password protection that is behind the hospital electronic firewall. Only the study investigators will have access to the database. If the results of the study are published, your name will not be used and no information that discloses your identity will be released or published. The samples will be stored in a laboratory area that is monitored by hospital security. All study data will be stored for 5 years.

### **Questions:**

If you have any questions about this study please contact Dr. Anthony Nichols at [redacted]. Dr. Nichols is a thyroid surgeon, and principal investigator in this study.

If you have questions about the conduct of this study or your rights as a research subject you may contact Dr. David Hill, Scientific Director at London Health Sciences Centre, at [redacted].

Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of this research.

A copy of this letter is for you to keep. Thank you for considering our request.

\_\_\_\_\_ Initials



Detection of Circulating Tumor DNA in thyroid nodular disease  
PI: Dr. Anthony Nichols  
Letter of Information and Consent for Papillary Thyroid Cancer Patients

I have read the Letter of Information/Consent Document, I have had the nature of the study explained to me, and I agree to participate. All questions have been answered to my satisfaction.

Printed Name of Participant: \_\_\_\_\_

Signature of participant: \_\_\_\_\_ Date: \_\_\_\_\_

Name of Person Obtaining Informed Consent:

- ☐ Dr. Anthony Nichols
- ☐ Dr. John Yoo
- ☐ Dr. Kevin Fung
- ☐ Dr. Danielle MacNeil
- ☐ Dr. Deric Morrison
- ☐ Dr. Stan Van Uum
- ☐ Dr. Terri Paul
- ☐ Dr. Irina Rachinsky

Signature of Person Obtaining Informed Consent: \_\_\_\_\_

Date: \_\_\_\_\_

# Appendix G: Letter of Information and Informed Consent



Otolaryngology - Head and Neck Surgery

Detection of Circulating Tumor DNA in thyroid nodular disease  
PI: Dr. Anthony Nichols  
Letter of Information and Consent

Letter of Information and Consent

Detection of Circulating Tumor DNA in Thyroid Nodular Disease

Principal Investigator: Dr. A. Nichols

Co-Investigators: Drs. J. Barrett, J. Mymryk, J. Yoo, K. Fung, D. MacNeil, D. Morrison, I. Rachinsky, S. Van Uum, T. Paul.

## Request for Participation:

You are being invited to participate in a research study to develop a blood test to identify cancer in thyroid nodules because you are over the age of 18 years and have been diagnosed thyroid nodules and have been booked for thyroid surgery, or have been diagnosed with papillary thyroid cancer and will be undergoing treatment for it. Patients who are below the age of 18 years or have had previous cancers that are known to be positive for mutations to be detected such as melanoma will be excluded from this study. This letter of information is provided for your review to obtain consent for your participation in the proposed study.

A team of scientists who specialize in thyroid research at the London Regional Cancer Program will analyse DNA in your blood sample to look for changes in DNA due to a potential thyroid cancer. This could lead to important information in the future about how to diagnose, treat and follow-up patients with thyroid nodules. This research requires samples from approximately four hundred patients in order to have enough information to produce meaningful results. If you agree to participate, information will be collected about your treatment, your follow-up visits to the clinic, and whether your thyroid contained cancer or not. Only the study investigators will have current and future access to this information.

## Participation Details:

### Blood Sample

As part of the routine work up for thyroid nodules, you will undergo thyroid blood tests before and after your operation. An additional 10 mL blood sample will be taken at the time of routine blood work before and at 3 months, 6 months and one year after the surgery. Just after the completion of the surgery, once the specimen is removed, an additional blood sample of 10 mL will be taken. All of these will be tested for specific mutations (genetic changes) that are thought to be correlated with thyroid cancer.

### Thyroid Sample

After the routine analysis of your thyroid is complete to determine if the cancer is benign or cancerous, a portion of the sample will be taken. DNA will be removed from the sample and undergo genetic testing. The genetic status of the tumor sample will be compared to the results of the blood testing.

## Results of Participation:

You do not need to spend any extra time participating in this study, besides the time needed for your treatment, routine blood work and your follow-up visits with your doctor. There is no risk involved with your participation in this study above the standard blood work.

\_\_\_\_\_  
Initials

Version 6.0 July 19, 2014







### Detection of Circulating Tumor DNA in thyroid nodular disease

PI: Dr. Anthony Nichols

#### Letter of Information and Consent

As mentioned previously, this is a preliminary study investigating the utility of detecting thyroid tumor DNA in a blood test. The detection will be done in a research lab that is not certified for medical purposes. Thus the results of the presence or absence of abnormal circulating thyroid tumor DNA will not be disclosed to you. You will most certainly be told about the pathology of your thyroid nodule at the time of your follow up and future treatment plan if any is required.

We are also asking for your permission to enter your medical information (including your age, tumour size, type of tumor) into a database so that we can keep track of your treatment, and outcome. The results from this research study may lead to valuable information including better treatment for thyroid disease. There may be no direct benefits to you for participating in this study, but the blood and tumor samples and information we collect may help other patients with thyroid disease in the future.

#### **Voluntary Participation:**

Participation in this study is voluntary. You may refuse to participate or withdraw from the study at any point with no effect on your future care. If you do withdraw from the study, any information collected up until that point will be kept in the database but no more information will be collected. The data collected must be retained in order to protect the integrity of research up to that point.

#### **Confidentiality:**

Every effort will be made to ensure that the confidentiality of your medical information is protected. Your medical information will be recorded in a secure computer database with password protection that is behind the hospital electronic firewall. Only the study investigators will have access to the database. If the results of the study are published, your name will not be used and no information that discloses your identity will be released or published. The samples will be stored in a laboratory area that is monitored by hospital security. All study data will be stored for 5 years.

#### **Questions:**

If you have any questions about this study please contact Dr. Anthony Nichols at [redacted]. Dr. Nichols is a thyroid surgeon, and principal investigator in this study.

If you have questions about the conduct of this study or your rights as a research subject you may contact Dr. David Hill, Scientific Director at London Health Sciences Centre, at [redacted].

Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of this research.

A copy of this letter is for you to keep. Thank you for considering our request.

\_\_\_\_\_ Initials



Detection of Circulating Tumor DNA in thyroid nodular disease  
PI: Dr. Anthony Nichols  
Letter of Information and Consent

I have read the Letter of Information/Consent Document, I have had the nature of the study explained to me, and I agree to participate. All questions have been answered to my satisfaction.

Printed Name of Participant: \_\_\_\_\_

Signature of participant: \_\_\_\_\_ Date: \_\_\_\_\_

Name of Person Obtaining Informed Consent:

<input type="checkbox"/>	Dr. Anthony Nichols
<input type="checkbox"/>	Dr. John Yoo
<input type="checkbox"/>	Dr. Kevin Fung
<input type="checkbox"/>	Dr. Danielle MacNeil
<input type="checkbox"/>	Dr. Deric Morrison
<input type="checkbox"/>	Dr. Stan Van Uum
<input type="checkbox"/>	Dr. Terri Paul
<input type="checkbox"/>	Dr. Irina Rachinsky

Signature of Person Obtaining Informed Consent: \_\_\_\_\_

Date: \_\_\_\_\_

## Appendix H: List of Abbreviations

ASIR	Age Standardized Incidence Rate
AUS	Atypia of Unknown Significance
cfDNA	Cell free DNA
ctDNA	Circulating Tumor DNA
FNAB	Fine Needle Aspirate Biopsy
FLUS	Follicular Lesion of Unknown Significance
FN	Follicular Neoplasm
FTC	Follicular Thyroid Cancer
LHSC	London Health Sciences Centre
PTC	Papillary Thyroid Cancer
PCR	Polymerase Chain Reaction
SFN	Suspicious for Follicular Neoplasm (or Hurthle Cell Neoplasm)
TSH	Thyroid Stimulating Hormone
US	Ultrasound
US-FNAB	Ultrasound guided Fine Needle Aspirate Biopsy



## Curriculum Vitae

Krupal Bhupendrabhai Patel MD, B.Sc

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### Education

<b>PGY4 Department of Otolaryngology – Head and Neck Surgery</b> Western University, London, ON	anticipated 2017
<b>Masters of Science in Surgery (Candidate)</b> Western University, London, ON	2014 – 2015
<b>Doctor of Medicine</b> College of Medicine, University of Saskatchewan	2012
<b>B.Sc (High Honours) Microbiology and Immunology</b> College of Arts and Science, University of Saskatchewan	2004 – 2008

### Awards and Grants

<b>C.A. Thompson Award for Scientific Achievement</b> Otolaryngology – Head and Neck Surgery 40 <sup>th</sup> Residents Research Mutational Landscape of Anaplastic Thyroid Cancer	2014
<b>Thyroid Cancer Foundation Grant</b> Patel K, Nichols A	2014
<b>Lawson Internal Research Fund “Pilot Studies” Grant</b> Patel K, Sowerby L Effect of low dietary salicylate on biochemical markers of Aspirin Exacerbated Respiratory Disease	2013
<b>Undergraduate Summer Research Projects Poster Competition</b> 2 <sup>nd</sup> Place – Medicine Category, College of Medicine, University of Saskatchewan	2010
<b>Dean’s Summer Research Award</b> College of Medicine, University of Saskatchewan	2009, 2010
<b>Saskatoon Health Region Foundation Grant</b> Patel K, Rosenberg A, Gerds V College of Medicine, University of Saskatchewan	2009
<b>NSERC Summer Student Research Award</b> University of Saskatchewan	2008

## PUBLICATIONS & PRESENTATIONS

### **Publications**

Patel K, Nicholas Cormier, John Barrett, John Yoo, Kevin Fung, Danielle MacNeil, Christopher Howlett, William Stecho, Nichols A. C. **Detection of Circulating Thyroid Tumor DNA in Patients with Thyroid Nodules.** (pending submission)

Patel, K. Barrett, J. W., & Nichols, A. C. **Review of Circulating Tumor DNA in Thyroid Cancer.** (pending submission)

Pinto, N., Black, M., Patel, K., Yoo, J., Mymryk, J. S., Barrett, J. W., & Nichols, A. C. (2014). **Genomically Driven Precision Medicine to Improve Outcomes in Anaplastic Thyroid Cancer.** *Journal of Oncology*, 2014

### **Presentations**

#### **Detection of Circulating Thyroid Tumor DNA in Patients with Thyroid Nodules – 2015**

Patel K, Nicholas Cormier, John Barrett, John Yoo, Kevin Fung, Danielle MacNeil, Christopher Howlett, William Stecho, Nichols A

Podium presentation: 41<sup>st</sup> annual Otolaryngology – Head & Neck Surgery Residents Research Day, Western University, London, ON

#### **Mutational Landscape in Anaplastic Thyroid Cancer – 2014**

Patel K, John Barrett, John Yoo, Kevin Fung, Danielle MacNeil, Nichols A

Podium presentation: 40<sup>th</sup> annual Otolaryngology – Head & Neck Surgery Residents Research Day, Western University, London, ON

### **Posters**

#### **Detection of Circulating Thyroid Tumor DNA in Patients with Thyroid Nodules – 2015**

Patel, K., Cormier, N., Barrett, J. W., Yoo, J., Fung, K., MacNeil, S. D., Howlett, C., Stecho, W., Nichols A. C.

Poster Presentation: Oncology Research Day, Western University, London, ON

#### **Survival of Patients with Subglottic Squamous Cell Carcinoma – 2015**

MacNeil, S. D., Patel, K., Liu, K., Shariff, S., Yoo, J., Nichols A. C., Fung, K., Garg A. X.

Poster Presentation: Canadian Society of Otolaryngology, Winnipeg, MB

#### **Intranasal Eosinophilic Angiocentric Fibrosis and Granuloma Faciale – a form of IgG4 related Disease – Case Report and Literature Review**

Patel K, Wehrli B, Moore C, Sowerby L

Poster presentation: 40<sup>th</sup> annual Combined Otolaryngological Society Meeting (American Rhinology Society)