Differentiated Thyroid Cancer – Evaluation of Contemporary Management and Novel Risk Assessment

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Differentiated Thyroid Cancer – Evaluation of Contemporary Management and Novel Risk Assessment

A thesis submitted to the National University of Ireland for the degree of

Doctor of Medicine (MD) in Surgery

by

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Dedication

This thesis is dedicated to Doreen Farrell.

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Publications

Differentiated thyroid cancer: How do current practice guidelines affect management?
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Communications

2017 Follicular variant papillary thyroid carcinoma in Western Europe: A Distinct Clinical Entity. Meeting of the British Associations of Endocrine and Thyroid Surgeons, Belfast.

2016 A polymorphism in the KRAS 3' UTR microRNA binding site: A case-control analysis assessing impact on differentiated thyroid cancer risk.
Joint Meeting of British and German Associations of Endocrine and Thyroid Surgeons, Berlin.

2016 A polymorphism in the KRAS 3' UTR microRNA binding site: A case-control analysis assessing impact on differentiated thyroid cancer risk.
European Society of Endocrine Surgeons, 7th biennial congress, Istanbul, Turkey. Accepted, but meeting cancelled due to political unrest.

2016 A polymorphism in the KRAS 3' UTR microRNA binding site: A case-control analysis assessing impact on differentiated thyroid cancer risk.
Plenary session - Irish Endocrine Society Meeting, Stormont, Belfast.

2016 Differentiated thyroid cancer: How do current practice guidelines affect management?
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2016 DTC susceptibility in a Western European population - A common variant at a 9q22 locus.
Plenary Session - 24th Sylvester O'Halloran Surgical Scientific Symposium, University of Limerick.

2015 FOXE1 – a potential thyroid cancer predisposition gene: A case-control study. Accepted for **BJS Plenary Session** - 35th Meeting of the British Associations of Endocrine and Thyroid Surgeons, Reading, UK.

2015 FOXE1 – a potential thyroid cancer predisposition gene: A case-control study.
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2015 Rs965513 increases susceptibility to differentiated thyroid cancer.
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2015 Investigating the role of polymorphism rs2910164 in mir146a in cancer predisposition.
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2015 Investigating the role of polymorphism rs2910164 in mir146a in cancer predisposition.
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2015 Differentiated Thyroid Cancer: Assessment of Clinical Practice in a Tertiary Referral Centre.
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2015 Differentiated Thyroid Cancer - Assessment of Clinical Practice in a Tertiary Referral Centre.
23rd Sylvester O'Halloran Surgical Scientific Symposium, UL
17th European Congress of Endocrinology, Dublin.

Abbreviations and Acronyms

AJCC	American joint committee on cancer
ANOVA	Analysis of variance
ATA	American thyroid association
BTA	British thyroid association
CT	Computed tomography
DNA	Deoxyribonucleic acid
DTC	Differentiated thyroid cancer
ESMO	European Society for Medical Oncology
USS	Ultrasound scan
EDTA	Ethylenediaminetetraacetic acid
EFVPTC	Encapsulated follicular variant of papillary thyroid cancer
FAP	Familial adenomatous polyposis
FNAC	Fine needle aspiration cytology
FNMTC	Familial non-medullary thyroid carcinoma
FTC	Follicular thyroid cancer
GDPR	General data protection regulation
GLOBOCAN	Global cancer incidence, mortality and prevalence
GWAS	Genome-wide association study
MAPK	Mitogen-activated protein kinase (pathway)
MDT	Multi-disciplinary team
MEN	Multiple endocrine neoplasia
MNG	Multi-nodular goitre
MiRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MTC	Medullary thyroid cancer
NCRI	National Cancer Registry Ireland
NFW	Nuclease-free water
NGS	Next-generation sequencing
NIFTP	Non-invasive follicular thyroid neoplasm with papillary-like nuclear features
NIS	Sodium iodine symporter
NMTC	Non-medullary thyroid cancer
NPV	Negative predictive value
OR	Odds ratio
PCR	Polymerase chain reaction
PET	Positron emission tomography
PPV	Positive predictive value
PTC	Papillary thyroid cancer
PTH	Parathyroid hormone
RCPath	Royal College of Pathologists, United Kingdom
RNA	Ribonucleic acid
RRA	Radioiodine remnant ablation
SNP	Single nucleotide polymorphism
TNM	Tumour, nodes, metastasis
TSAb	Thyroid-stimulating antibody
TSH	Thyroid stimulating hormone
UHG	University Hospital Galway
UTR	Untranslated region

Abstract

Introduction

Differentiated thyroid cancer (DTC) is a heterogeneous disease with a spectrum of phenotypes ranging from indolent to aggressive variants. The incidence of DTC has increased by 300% over the past 15 years in Ireland. Current best practice guidelines recommend a "personalised decision making" approach for a large cohort of patients with intermediate size tumours where there is a paucity of sufficient quality evidence to support specific recommendations for their management. This cohort may be over treated due to equivocal guidelines. An improved understanding of the molecular mechanisms of thyroid tumourigenesis, and knowledge of low risk mutant alleles which contribute to DTC susceptibility and impact disease phenotype may improve our ability to individualise treatment for these patients.

We ascertain the effect of equivocal guidance, particularly for patients with intermediate size 1-4cm tumours, on contemporary practice patterns. With a view to improving risk stratification for those subject to equivocal guidelines, we validate the effect of single nucleotide polymorphisms in genes affecting thyroid biology for patients with DTC, and investigate the role of these genotypes in modifying DTC risk. We also examine the impact of mutations in miRNA, or in miRNA-mRNA binding sites on DTC susceptibility and phenotype.

Methods

Patients were recruited from thyroid cancer treatment clinics at tertiary centres in the West of Ireland and South of France as part of a collaborative multicentre study to establish a thyroid cancer biobank at the Discipline of Surgery in the Lambe Institute for Translational Research, based in University Hospital Galway. Patient demographics and clinico-pathological data were recorded. Controls were recruited from community volunteers and were >60 years old, without a current or previous diagnosis of cancer, and without a first-degree family history of thyroid cancer.

Patterns of DTC presentation and therapeutic approaches were assessed and compared to current British Thyroid Association clinical practice guidelines. We ascertained those subgroups subject to equivocal guidance.

Germline DNA was extracted from blood or buccal swabs using crystallisation precipitation, and genotyped using Taqman-based PCR. A case–control analysis was undertaken, comparing genotypic and allelic frequencies of the FOXE1 variant in patients with DTC, to frequencies in unaffected

controls. Three miRNA-associated mutations (miR146a, KIT and KRAS) were also genotyped among cases and controls, and the effect on DTC susceptibility assessed.

Results

Of 178 patients assessed, an equivocal "personalised decision making" approach was the recommended strategy for 32 and 80 patients for surgery and RRA respectively; almost all proceeded to the more aggressive options of completion surgery and RRA.

277 patients with confirmed DTC and 309 non-cancer controls were genotyped for the FOXE1 variant. We demonstrated a significant association between the mutation and DTC susceptibility. An allele dosage effect was observed with odds ratios (OR) of 1.66 and 2.93 for heterozygous and rare homozygous genotypes respectively. Amongst Irish patients alone, presence of the rare homozygous genotype conferred an odds ratio of 3.9 ($p<0.00001$) for DTC risk.

Three miRNA-associated variants were assessed for their impact on DTC susceptibility. The miR146a variant exhibited a per allele OR of 1.64 ($p<0.001$) for DTC cases (n=175), compared to controls (n=637). For those with one copy of the mutant allele at the locus, a genotypic OR of 1.62 (p=0.008) was observed, rising to 2.51 (p=0.006) for rare homozygous carriers.

The KIT variant rs17084733 is associated with a reduced risk for development of DTC. The variant allele was evident in 54% of cases (n=275) and 61% of controls (n=440), with an associated per allele odds ratio of 0.65 (p=0.012). Rare homozygous carriers exhibited a lower genotypic OR compared to heterozygous carriers (OR=0.72, p=0.077 vs OR=0.24, p=0.046).

The minor allele frequency of a novel germline T>G polymorphism in the 3' UTR of KRAS was observed to be 8% for both cases (n=274) and controls (n=669). No significant association was evident between presence of the mutant allele and DTC susceptibility (per allele OR=1.03, p=0.869).

Conclusions

Current best practice guidelines for the management of patients with DTC do not adequately delineate the appropriate management for large subgroups of patients for which there is a paucity of high quality evidence to guide clinical practice. This work demonstrates the significant extent to which patients treated in tertiary referral centres are subject to equivocal guidelines.

Through genotyping studies we have demonstrated a significant impact on DTC risk associated with a germline mutation in the FOXE1 gene. Furthermore we have elucidated the effects of three miRNA-associated variants on DTC susceptibility, with the mir146a and KIT variants exhibiting a significant impact on DTC susceptibility. Testing for these variants should be considered as part of a gene mutation panel to improve DTC risk stratification for patients with this common malignancy.

1 Chapter 1 – Introduction

1.1 Thyroid cancer epidemiology

The incidence of cancer, including thyroid malignancy, is increasing worldwide.[1] National Cancer Registry Ireland (NCRI) forecast that the incidence of cancer has increased such that, one in every two people born since 1960 will get a cancer diagnosis in their lifetime and by 2040, based on recent incidence trends and changes in population demographics, cancer incidence in Ireland is expected to increase by up to 125% for females and 133% for males.[2]

Thyroid cancer, although a relatively rare disease, is the most common endocrine malignancy. The most recently reported NRCI data for thyroid cancer reports a mean of 265 cases diagnosed per year from 2010 to 2015, an increase from 162 per year from 2006 to 2010; this equates to an incidence of approximately 5.5 per 100,000. Thyroid cancer is predominantly a disease of young females, with lifetime risk of diagnosis being 2.5 times higher in females (1 in 240) compared to males (1 in 580), being most commonly diagnosed between the ages of 30 and 49 (Figure 1).[3]

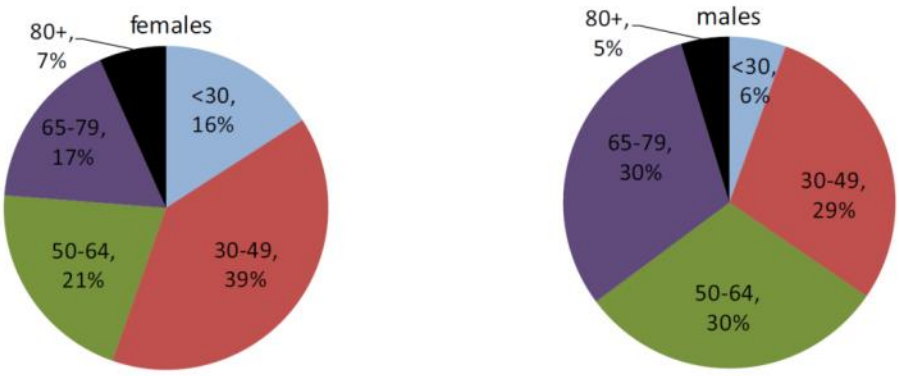


Figure 1: Gender specific age distribution of thyroid cancer in Ireland (1994-2010) (Image reproduced from NCRI)[3]

The major histological subtypes of thyroid cancer are papillary, follicular, medullary, anaplastic and poorly differentiated. Together, the papillary and follicular subtypes are known as differentiated thyroid cancer, accounting for approximately 80% of all thyroid malignancies, and are the focus of this thesis.[3] Anaplastic and poorly differentiated tumours, while also originating in follicular epithelium, are associated with a far more aggressive disease phenotype, while medullary tumours arise from the parafollicular c-cells of the thyroid gland and are a distinct histological entity.[4,5]

Histological subtype varies by age and gender, with well-differentiated subtypes being more common in younger females. In particular, papillary tumours demonstrate the greatest age-related variance, accounting for nearly 80% of subtypes for those younger than 30 years, decreasing to only 20% of tumours in patients greater than 80 years of age (Figure 2).[3]

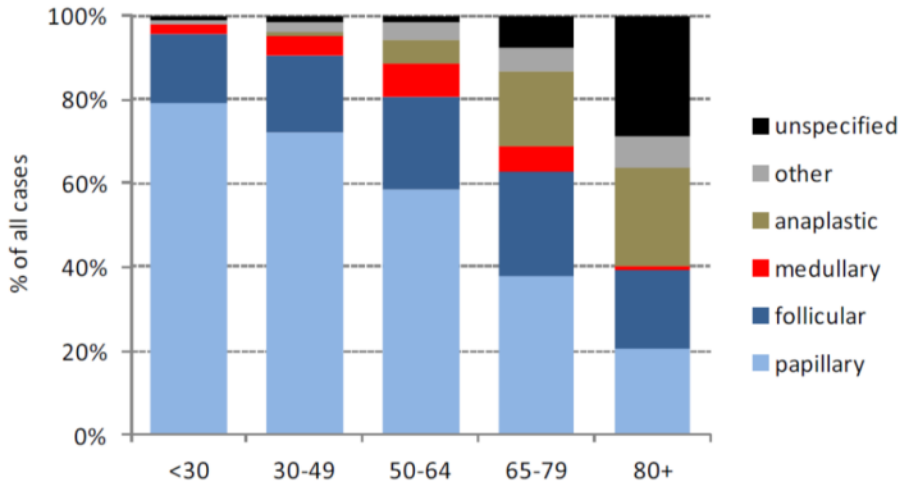


Figure 2: Thyroid cancer subtype variation by age (1994-2010) (Image reproduced from NCRI)[3]

The incidence of thyroid cancer diagnosis varies significantly with geographical location, with some reports of a greater than 10 fold differential in incidence between countries.[6] International comparisons are difficult due to variations in data collection, diagnostics and screening protocols. Notwithstanding this, the highest rates of incidence globally are reported in South Korea, the Pacific islands, Japan, Hawaii and in Europe, Lithuania, France, Italy and Croatia. In general, incidence rates are approximately double for high-income countries compared to low- and middle-income countries.[6]

There has been a significant increase in the global incidence of thyroid cancer in recent decades, particularly among females.[1] This trend is also observed in Ireland where incidence rates have increased from approximately 40 females and 20 males per year in the mid 1990's to 120 females and 45 males in the late 2000's. The majority of these additional cases are comprised of small well-differentiated tumours, usually papillary subtypes, and to a lesser degree, follicular tumours (Figure 3).[3] Similar figures have been reported internationally with thyroid cancer forecast to replace colorectal cancer as the fourth most commonly diagnosed cancer in the United States by 2030.[7-10] The precise reasons for recent increases in thyroid cancer diagnoses are not fully

understood, however, increased use of diagnostic modalities such as computed tomography (CT), ultrasound scanning (USS) and fine-needle aspiration cytology (FNAC) may be largely responsible. This explanation is supported by the absence of a corresponding rise in thyroid cancer mortality rates and the increasing rates of thyroid micropapillary tumour diagnoses. The mortality from thyroid cancer is relatively low (12 deaths per annum in Ireland) and is either static or slowly decreasing globally in spite of the current thyroid cancer 'epidemic' (Figure 4).[3,6,9,11] A number of factors may contribute to the global decrease in disease mortality including a reduction in benign thyroid disease secondary to reducing iodine deficiency and endemic goitre prevalence internationally; and more targeted and restrained use of ionising radiation in medical treatments, particularly in children.[6]

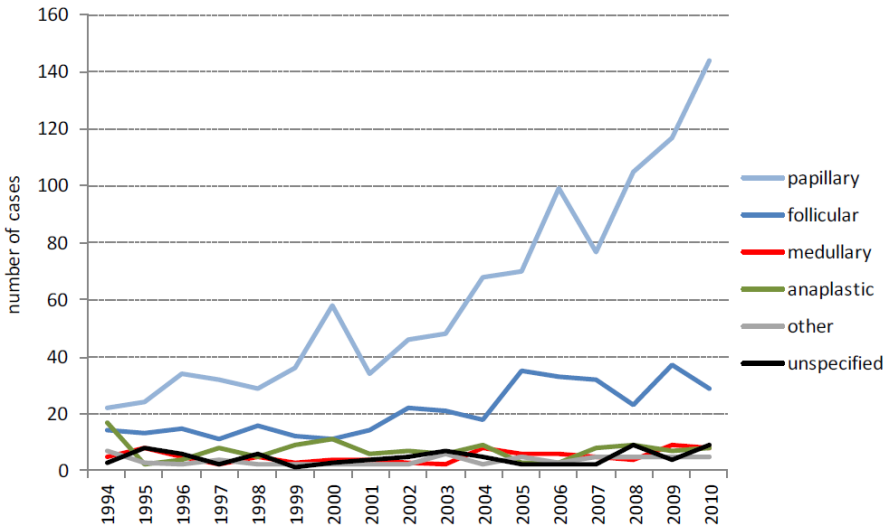


Figure 3: Increasing incidence of thyroid cancer by subtype in Ireland (1994-2010) (Image reproduced from NCRI)[3]

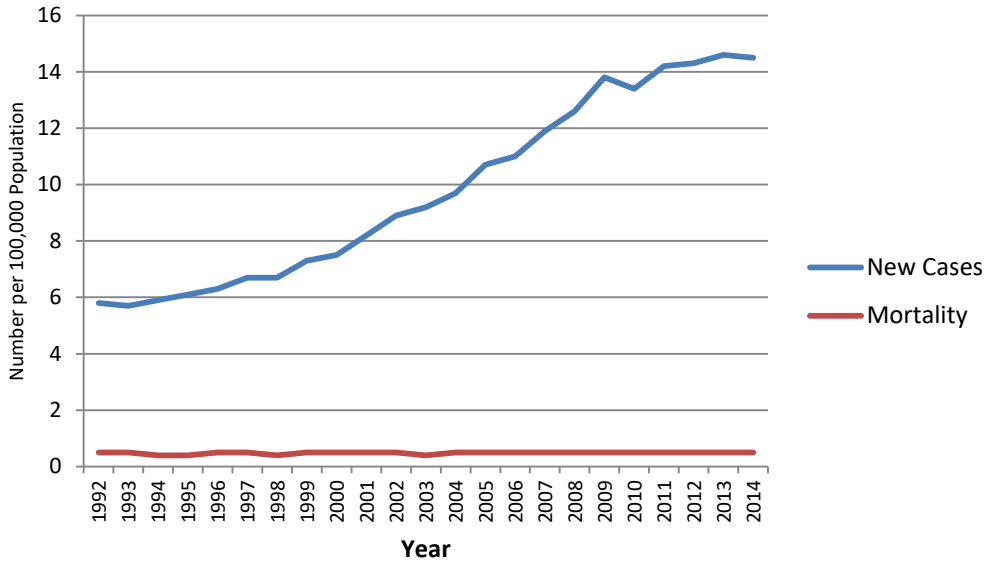


Figure 4: Cases and deaths per 100,000, age-adjusted, SEER USA (1992-2014) (Image reproduced from NCRI)[11]

The concept of over-diagnosis is pertinent to the topic of thyroid cancer given the global increase in DTC incidence over recent decades. Numerous definitions of over-diagnosis can be found in the literature and as a result, significant variation exists when attempting to quantify the extent of over-diagnosis with respect to a given condition. Two definitions describe over-diagnosis as individuals receiving a diagnosis with a condition that would never have become symptomatic before the end of the individual's life, or alternatively, giving a diagnosis for which treatment will not yield a net benefit.[12] Over-diagnosis can have significant implications, both for patients and healthcare organisations, including physical, psychosocial and financial sequalae arising from unnecessary investigation and treatment. The main causes of over-diagnosis are over-detection, and over-definition of disease, both of which are relevant to the increasing burden of DTC globally.[13] Over-detection addresses the growing incidence of disease secondary to increased access to healthcare and disease screening, and the improved sensitivity and diagnostic accuracy of high definition imaging, whereas over-definition of disease refers to over-medicalisation of what may be considered benign or normal-variant pathologies.[13] With reference to DTC, these causes of over-diagnosis are illustrated by the improved sensitivity of USS in the detection of thyroid nodules and the reported overtreatment of indolent DTC subtypes such as encapsulated follicular variant of PTC (EFVPTC), which has recently been reclassified as a non-malignant entity based on a multicentre consensus study, and is now known as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP).[14,15]

The potential role of over-diagnosis in the increasing burden of thyroid cancer is illustrated well in two contrasting case studies – Republic of South Korea and Fukushima, Japan. In South Korea, an USS based thyroid screening programme was introduced in the early 2000's, with the incidence of thyroid cancer thereafter increasing by more than seven-fold from 6.3 per 100,000 in 1999, to 47.5 per 100,000 in 2009. This change was accompanied by a proportional increase in economic burden from €232m to €1,556m, approximately 1.2% of the national yearly healthcare expenditure, with no accompanying improvement in thyroid cancer mortality rates during the same period.[16-18] Park et al conclude that this 'epidemic' is almost entirely due to over-diagnosis from widespread use of sensitive imaging tools. However, some commentators are slower to exclude other unidentified causes such as radiation exposure from nuclear power plants or evolving risk factors yet to be elucidated.[16] These cautions are supported by a number of other factors which suggest that more than over-diagnosis may account for the observed increase; these include a documented increase in not only small incidental tumours, but also larger symptomatic lesions, albeit to a lesser extent; and disproportionate increases in papillary histological subtypes, tumours in females and in younger age groups.[19,20] In Fukushima, Japan, an USS screening programme was introduced for residents younger than 18 years following an incident at the Fukushima Daiichi nuclear power plant in the wake of the East Japan earthquake and tsunami in March 2011. Interim data from the screening programme of 298,577 individuals, published by Toshihide Tsuda et al, demonstrated an approximately 30-fold increase in thyroid cancer cases compared to the baseline Japanese national incidence 4 years post-event.[21] One concern noted in this study is that although ionising radiation is likely to be a main driver of this excess thyroid cancer incidence, widespread screening may also have contributed, particularly given an increase in incidence which exceeded that forecast by the World Health Organisation (WHO), in addition to the short timeframe of 4 years, which may be too rapid for the onset of radiation induced thyroid cancer.[21]

Although the literature tends towards a consensus that the increasing incidence of thyroid cancer is predominantly due to 'over-diagnosis' of the disease, many believe it to be a multifactorial phenomenon, and therefore it is prudent not to dismiss genetic, environmental or lifestyle factors which remain to be elucidated.[6,8,22-26] Whatever the aetiology, there remains a clinical challenge for those involved in the care of patients with thyroid cancer; we must endeavour to optimise risk stratification for this increasingly large patient cohort in order to appropriately manage the individual patient and avoid overly aggressive treatments and surgical interventions giving rise to potential physical and psychological morbidities for patients, and resource implications for healthcare systems.

1.2 Risk factors

A number of modifiable and non-modifiable risk factors are associated with the development of thyroid cancer, disease progression and recurrence. In light of the debate surrounding the increases in thyroid cancer incidence globally, there is much interest in better understanding these risk factors and rationalising their use in risk stratification of patients before, during and after the diagnosis of thyroid cancer. Non-modifiable risk factors for development of thyroid cancer include female gender, age, ethnicity, family history, and a number of predisposing hereditary conditions and genetic mutations, while the strongest known modifiable risk predictor is exposure to ionising radiation, particularly in childhood. Personal or family history of other cancers and benign thyroid disease are also strongly associated with an elevated risk for thyroid malignancy.[27,28] Other factors such as smoking, obesity and ethnicity are possible risk factors but remain to be demonstrated conclusively.[29]

1.2.1 Gender

In Ireland, thyroid cancer is 2.5 times more common in females than males.[3] Similar gender preponderance is evident in other nationalities, although to varying degrees, and has been changing in recent decades with a widening disparity. The 5th GLOBOCAN database report, published in 2012 and covering 184 countries worldwide, reports approximately 230,000 female thyroid cancer cases compared with only 68,000 male cases, giving rise to a female to male ratio of 3.4 : 1.[10] This compares to their equivalent 2002 report which documented a female to male ratio of 2 : 1.[30] Although thyroid cancer is more common in females, it is more likely to present at an earlier disease stage, with less aggressive subtypes and a reduced risk of mortality. More aggressive subtypes of thyroid cancer such as medullary and anaplastic, display less gender disparity, while DTC of follicular cell origin i.e. papillary and follicular, are more common in women.[31-33]

1.2.2 Age

Thyroid cancer tends to be diagnosed at a younger age than many other malignancies, with females having a younger profile than males. In Ireland, from 1998 to 2007, the median age of diagnosis was 45 years for women and 52 years for men, while 55% of females, but only 35% of males, were diagnosed below the age of 50 (Figure 1).[34] In the United States, The Surveillance, Epidemiology, and End Results (SEER) program reports the median ages for thyroid cancer diagnosis, between 1992 and 2012, were 49 and 54 years for females and males respectively.[11]

1.2.3 Ethnicity

Traditionally, thyroid cancer incidence has been reported as higher in non-Caucasians, in particular Asians and Pacific Islanders.[35,36] Although ethnicity data is limited for Ireland, Finlayson et al report in the British Journal of Cancer that between 2001 and 2007, thyroid cancer incidence in the UK was significantly higher in all ethnic groups compared to Caucasians, in particular for Pakistanis (RR 1.7), Africans (RR 1.7) and Chinese (RR 2.1) populations.[36] However, recent data from the US SEER 13 database noted that following disparate ethnic changes in thyroid cancer incidence, thyroid cancer incidence has now become higher in Caucasian populations in the USA since 2002, than for Asians and Pacific Islanders.[35]

1.2.4 Familial/genetic risk

Thyroid cancer is known to have a strong familial component, although the mechanism is not yet clear for non-medullary subtypes. Published data suggests that the risk of developing non-medullary thyroid cancer is estimated to be approximately 3 to 6 times higher for people with a first degree relative with thyroid cancer, than for the general population. This relative risk tends to be higher for siblings, particularly sisters and for twins. For papillary tumours, standardised incidence ratios of 3.2 and 6.2 were reported for persons with parents and siblings with thyroid cancer respectively, while a female with an affected sister has a standardised PTC incidence ratio of 11.2.[37] Significantly increased relative risks have been demonstrated for relatives as distant as third-degree from thyroid cancer probands.[37-40]

A number of hereditary conditions are associated with increased risk for development of thyroid cancer, in particular, medullary thyroid cancer, which arises from the parafollicular cells (C cells). Approximately 1 in 4 cases of medullary thyroid cancer (MTC) are familial and are associated with germline point mutations in the 'rearranged during transfection' (RET) proto-oncogene. Familial MTC may occur in isolation, or as part of multiple endocrine neoplasia syndromes 2a or 2b, which are also commonly associated with phaeochromocytomas, parathyroid hyperplasia, mucosal neuromas and a marfanoid body habitus.[41]

In contrast to MTC, relatively little is known about the genetics of the remaining subtypes, particularly DTC. Although most non-medullary thyroid cancers (NMTC) occur spontaneously, a familial aetiology is thought to account for 5 – 10% of those cases with follicular cell origin.[42] Familial non-medullary thyroid cancers (FNMTCs) may occur in isolation or be associated with other syndromes, which are frequently associated with preponderance for development of other non-thyroidal neoplasms. These syndromes include Cowden's syndrome, familial adenomatous

polyposis (FAP), PTEN-hamartoma tumour syndrome (PHTS), Carney's complex type 1, and Werner's syndrome.[42]

The identification of novel genetic markers for DTC susceptibility is being increasingly investigated and is the subject of much of this thesis.

1.2.5 Ionising radiation

The thyroid gland is radiation-sensitive, with exposure to ionising radiation, particularly in childhood and adolescence, being a well-documented risk factor for thyroid tumourigenesis through somatic mutations.[43-45] Multiple pooled analyses of childhood radiation exposures demonstrate increased incidence of thyroid nodules of all sizes, with risk increasing in a dose-dependent fashion, while being inversely proportional to age of exposure.[46-48] Effects are noted for all tumour types, but most pronounced for papillary cancers and have been documented following a range of exposure types including atomic bomb fallout, radiation treatments for thymic hyperplasia and cancers, and accidental exposures. The estimated excess relative risk conferred per Gray of ionising radiation exposure has been estimated at 2.76 per year compared to non-exposed subjects, with this value remaining statistically significant up to 10 years post exposure, and remaining elevated for a further 40 years.[48] The effects of lower-dose radiation exposure (< 0.1 Gray) on the thyroid gland is not well established, giving rise to uncertainty surround the risks associated with occupational and medical exposure.[44]

1.2.6 Benign thyroid disease

Benign thyroid disease is also a significant risk factor for thyroid neoplasia. Both solitary nodules and multi-nodular goitres are associated, although other benign thyroid disease processes also confer increased risk.[28,49,50] While thyroid nodules do carry an increased chance for development of thyroid cancer, approximately 90% of nodules do not develop into cancers.[41,51] 5% of the general population are likely to have a clinically palpable thyroid nodule at a given time, while nodules can be demonstrated on USS in up to 70% of the population.[52] Part of the challenge in managing the increasing burden of thyroid cancer involves rationalising the extent of investigations and interventions undertaken for incidental thyroid nodules. A personal history of hypo- and hyper-thyroidism, Hashimoto's thyroiditis, endemic goitre and Graves' Disease have all been implicated in increasing the risk of thyroid cancer.[50,52-56] Thyroid stimulating hormone (TSH, thyrotropin), a growth factor for the thyroid gland, is commonly elevated in patients with goitre secondary to iodine

deficiency and in those with Hashimoto's thyroiditis. Both of these patient groups have an increased risk for development of thyroid cancer, thought to be mediated in part by excess TSH.[19] Conversely, TSH suppression (<0.1 mU/l) may reduce thyroid tumourigenesis; administration of high dose levothyroxine (LT4) achieves this, and has be shown by meta-analysis to reduce adverse outcomes in selected high-risk patients with differentiated thyroid cancer as part of post-operative management.[20,52,57,58] Graves' hyperthyroidism also confers an increased risk for thyroid cancer, likely mediated by thyroid-stimulating antibodies (TSAb), rather than elevated TSH which is typically low in Graves' disease.[55,59,60]

1.2.7 Other risk factors

A number of other modifiable risk factors have been examined in relation to thyroid tumourigenesis although the evidence remains weak or inconclusive. Thyrocarcinogenic factors may include obesity and insulin resistance, chronic hepatitis C, increased parity or advanced age at initial pregnancy, dietary nitrates, cruciferous vegetable, prolonged multi-vitamin use and environmental pollutants such as polybrominated diphenyl ethers (PDBEs).[19,61-64] Consumption of iodine rich foods, tea, wine and cigarette smoking, have been associated with a reduced thyroid cancer risk.[29,64,65]

1.3 Thyroid gland anatomy

The thyroid gland is a butterfly shaped solid organ situated in the anterior neck comprised of two lateral lobes joined by an isthmus which crosses anterior to the second and third tracheal cartilages (Figure 5).[66]

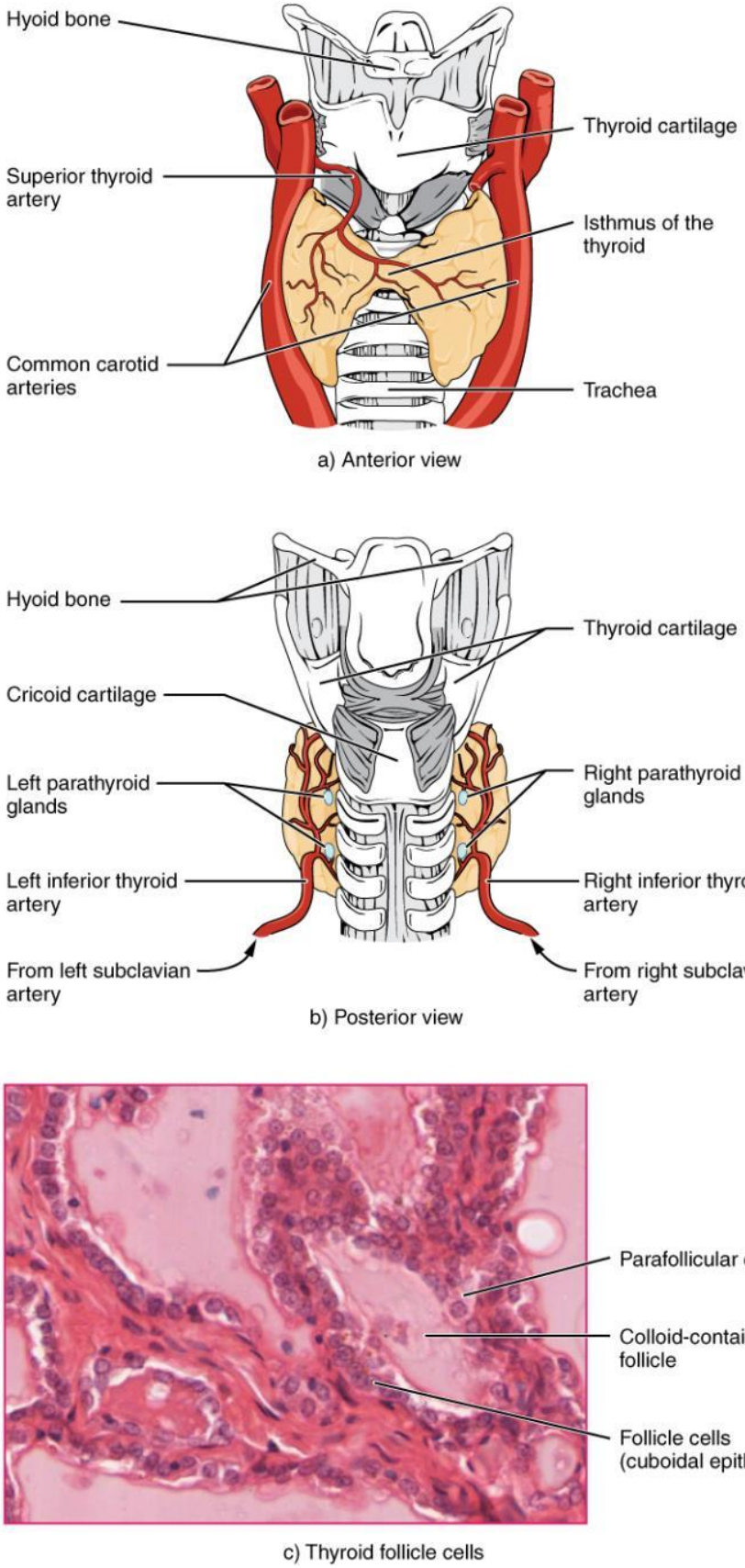


Figure 5: Thyroid Gland a) Anterior view b) Posterior view c) 100 x magnification H&E stain of typical thyroid follicles, colloid and interspersed parafollicular C-cells. (Image reproduced from Anatomy & Physiology)[67]

The gland is typically supplied by arterial branches of the external carotid and subclavian arteries while venous drainage is usually via tributaries to the internal jugular and brachiocephalic veins. Lymphatic drainage of the gland is predominantly to the paratracheal nodes (Level 6), but also to deep cervical nodes along the internal jugular vein. Knowledge of the lymphatic drainage and levels of neck dissection, as designated by the American Academy of Otolaryngology, Head and Neck Surgery, are crucial to achieving appropriate neck dissection and disease clearance in thyroid cancer patients where indicated (Figure 6).[68]

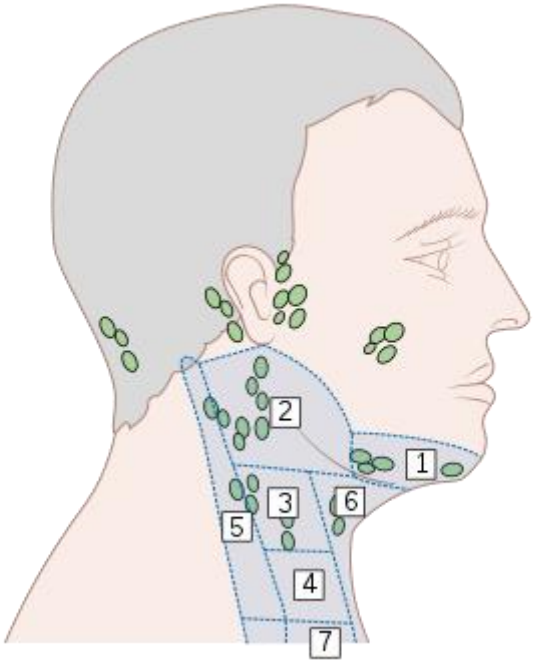


Figure 6: Head and neck lymph node groups and levels (Image reproduced from Cancer Research UK)[69]

Importantly, the thyroid gland is closely related to the recurrent laryngeal nerves, branches of the vagus nerve, which pass deep to the posteromedial surface of the lateral lobes of the thyroid gland and are at risk of injury during thyroidectomy. The parathyroid glands, which are responsible for parathyroid hormone (PTH) production, are closely related to the posterior aspect of thyroid gland and are at risk of excision or injury during surgery, with potential risk of subsequent hypocalcaemia.[66]

1.4 Thyroid cancer pathology and staging

Many neoplasms arise in the thyroid gland, which may be broadly classified as benign or malignant. Solitary nodules, as opposed to diffuse thyroid gland nodularity and goitres, while more likely to be neoplastic, are usually benign, comprising follicular adenomas, nodular hyperplasia, simple cysts, or foci of thyroiditis. Less than 1% of solitary thyroid nodules are malignant carcinomas, typically originating from thyroid gland epithelial cells. The thyroid gland contains two main cell types, follicular cells and parafollicular cells (C-cells) (Figure 5). The major histological subtypes of thyroid cancer are papillary, follicular, medullary, anaplastic and poorly differentiated. Together, the papillary, follicular, anaplastic and poorly differentiated tumour subtypes represent over 90% of thyroid malignancies and arise from follicular epithelium, while medullary thyroid cancers arise from the parafollicular c-cells of the thyroid gland.[4,5] Poorly differentiated and anaplastic carcinomas are relatively rare entities that, while originating in follicular epithelium, are associated with a far more aggressive disease phenotype than the well-differentiated PTC and FTC. Medullary thyroid carcinoma also represents a separate clinical and histological entity with distinct biologic features. This thesis focuses on the differentiated thyroid cancers (PTC and FTC).

1.4.1 Thyroid adenomas

Thyroid adenomas are the most common thyroid neoplasms, with an incidence ranging from 20-76% in autopsy and ultrasound studies.[70-72] These typically tumours arise from follicular epithelium and are usually solitary. Solitary nodules are predominantly of importance due to the need to exclude malignancy when detected; the prevalence of malignancy within thyroid nodules is up to 6.5% and 2% of thyroid malignancies arise within a previously benign adenoma.[73,74]

1.4.2 Pathology of differentiated thyroid cancer

Differentiated thyroid cancer (DTC) is comprised of two main histological subtypes, papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), each of which can be further divided into a number of variants (Table 1). DTCs arise in thyrocytes and express the sodium iodine symporter (NIS).[75]

Histology	Histological Variants
Papillary carcinoma	Classic (usual)
	Clear cell variant
	Columnar cell variant
	Cribriform-morular variant
	Diffuse sclerosing variant
	Follicular variant
	Macrofollicular variant
	Microcarcinoma (occult, latent, small, microtumour)
	Oncocytic or oxyphilic variant (follicular/nonfollicular variant)
	Solid variant
	Tall cell variant
	Warthin-like variant
Follicular carcinoma	Clear cell variant
	Oncocytic (Hürthle cell) variant
	Mucinous variant
	With signet-ring cells

Table 1: Histologic subtypes of a) Papillary thyroid cancer, and b) Follicular thyroid cancer[75,76]

PTCs may be solitary or multifocal tumours and are often encapsulated lesions. Variants without a capsule tend to infiltrate the adjacent parenchyma with ill-defined margins. They are usually comprised of a typical papillary architecture, contain areas of fibrosis and calcification, and are often cystic. Concentrically calcified structures termed psammoma bodies are often present within the papillae. The diagnosis of papillary carcinoma is predominantly based on nuclear features which include “ground-glass" or "Orphan Annie eye" nuclei, and cytoplasmic involutions giving the appearance of intranuclear inclusions or grooves on cross-section (Figure 7).

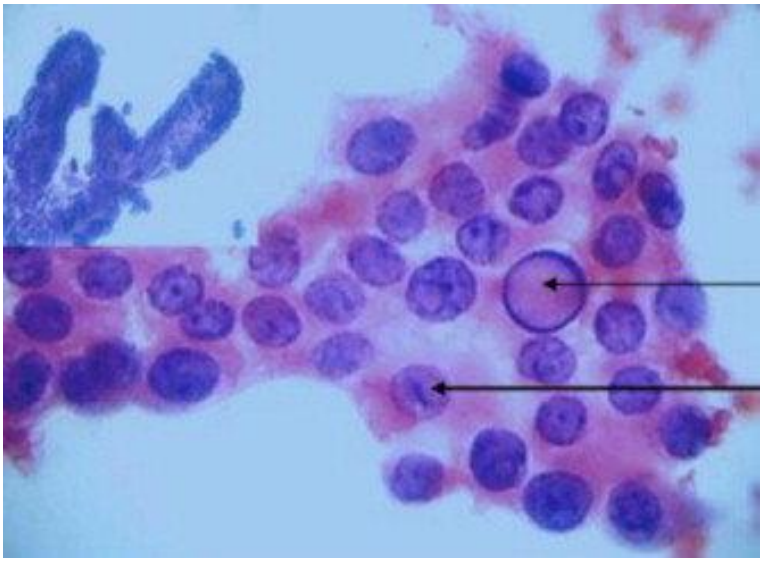


Figure 7: Photomicrograph (x 400) demonstrating cytological features of PTC including a ground glass 'orphan Annie eye' nucleus (thin arrow) and nuclear groove (thick arrow) (Image reproduced from Sinna)[77]

The common 'follicular variant' PTC (FvPTC), although composed predominantly of follicles rather than the classical papillary architecture, have the same nuclear phenomena and phenotypic behaviour as classical PTCs and are therefore classified as such. PTCs are rarely seen to invade blood vessels; lymphatic invasion and spread is far more common and is present in approximately 50% of cases.[4]

FTCs are usually solitary tumours composed of typically uniform cells in a follicular architecture and are frequently encapsulated. They range from minimally invasive and morphologically similar to follicular adenomas, to widely invasive lesions. Careful sampling of any tumour capsule is required to distinguish follicular adenomas from invasive follicular carcinomas. FTCs do not demonstrate the same nuclear characteristics as PTCs, being diagnosed predominantly based on histological architecture. This poses a problem for diagnosis where FNA cytology can often fail to demonstrate the difference between a follicular adenoma and an FTC, thereby necessitating diagnostic lobectomy in many cases. In contrast to PTCs, FTCs usually metastasise in a haematogenous manner, to lung, bone and liver with regional nodal spread being less common.[4]

Hürthle cell carcinomas, also known as oxyphilic cell or oncocytic thyroid carcinomas, are historically considered the main variant of the classical follicular carcinoma. Their typical oncocytic cells are defined by cellular enlargement with abundant eosinophilic granular cytoplasm due to accumulated altered mitochondria and are frequently seen in inflammatory conditions such as thyroiditis. Hürthle

cell neoplasms demonstrate hyper cellularity and are typically composed of greater than 75% Hürthle cells, with minimal colloid and without lymphocytes.[78,79]

1.4.3 Staging of differentiated thyroid cancer

The 8th edition of the AJCC/TNM cancer staging system was published in October 2016 for implementation from January 2018 (Tables 2 & 3).[80] The TNM staging system is designed to predict survival of patients with thyroid cancer and maintains the basic T-N-M approach used in the 7th Edition.[81]

	Age at diagnosis < 55 years			*10 year disease specific survival*
Stage I:	any T	any N	M0	98-100%
Stage II:	any T	any N	M1	85-95%
	Age at diagnosis ≥ 55 years			
Stage I:	T1	N0 / NX	M0	98-100%
	T2	N0 / NX	M0	98-100%
Stage II:	T1	N1	M0	85-95%
	T2	N1	M0	85-95%
	T3a / T3b	any N	M0	85-95%
Stage III:	T4a	any N	M0	60-70%
Stage IVA:	T4b	any N	M0	<50%
Stage IVB:	any T	any N	M1	<50%

Table 2: AJCC prognostic stage grouping for DTC[80]

Primary tumour (pT):
TX: Primary tumour cannot be assessed
T0: No evidence of primary tumour
T1: Tumour ≤ 2 cm in greatest dimension limited to the thyroid
T1a: Tumour ≤ 1 cm in greatest dimension limited to the thyroid
T1b: Tumour > 1 cm but ≤ 2 cm in greatest dimension limited to the thyroid
T2: Tumour > 2 cm but ≤ 4 cm in greatest dimension limited to the thyroid
T3*: Tumour > 4 cm limited to the thyroid or gross extra-thyroidal extension invading only strap muscles
T3a*: Tumour > 4 cm limited to the thyroid
T3b*: Gross extra-thyroidal extension invading only strap muscles (sternohyoid, sternothyroid, thyrohyoid or omohyoid muscles) from a tumour of any size
T4: Includes gross extra-thyroidal extension into major neck structures
T4a: Gross extra-thyroidal extension invading subcutaneous soft tissues, larynx, trachea, oesophagus or recurrent laryngeal nerve from a tumour of any size
T4b: Gross extra-thyroidal extension invading prevertebral fascia or encasing carotid artery or mediastinal vessels from a tumour of any size
Regional lymph node (pN):
NX: Regional lymph nodes cannot be assessed
N0: No evidence of regional lymph node metastasis
N0a*: One or more cytologic or histologically confirmed benign lymph nodes
N0b*: No radiologic or clinical evidence of locoregional lymph node metastasis
N1*: Metastasis to regional nodes
N1a*: Metastasis to level VI or VII (pretracheal, paratracheal, prelaryngeal / Delphian or upper mediastinal) lymph nodes; this can be unilateral or bilateral disease
N1b*: Metastasis to unilateral, bilateral or contralateral lateral neck lymph nodes (levels I, II, III, IV or V) or retropharyngeal lymph nodes
Distant metastasis (M):
M0: No distant metastasis
M1: Distant metastasis

Table 3: AJCC TNM Staging Guide (8th Ed) for DTC[80]

TNM staging is typically performed pre-operatively using clinico-radiological methods such as clinical examination and imaging studies including ultrasound, computed tomography and PET-CT where indicated. Pathologic staging (pTNM) is based on all information used for clinical staging plus histologic examination of the resected specimen post-operatively, in addition to the surgeon's description of the intra-operative findings. Accurate preoperative staging is crucial for patients and the multi-disciplinary team when choosing the optimal treatment pathway for patients with thyroid cancer.

A number of significant changes have been implemented in this updated AJCC / TNM 8th edition for thyroid cancer.[81] The effect of these changes will be to better predict survival in patients with DTC and increase the number of patients in the lower and intermediate risk stages of disease, thereby reducing the volume of high risk patients to less than 10% of patients with DTC. Implementation of these revised AJCC TNM staging guidelines should result in the overall down-staging of approximately a third of patients with thyroid cancer overall in an effort to avoid over-treating patients with potentially indolent disease.[81,82] The most noteworthy amendments include:

- Staging age cut-off increased from 45 to 55 years at diagnosis.
- Non-gross extra-thyroidal extension (i.e. evident only on histopathological examination only) now does not constitute T3 disease, and in fact only gross extra-thyroidal extension has any impact on T- or overall stage.
- N1 disease does not constitute stage 3 disease in this edition. Patients with N1 disease < 55 years at diagnosis will be stage 1 overall, while patients with N1 disease ≥ 55 years will be classed as stage II.
- T3a is a new category for tumours > 4 cm confined to the thyroid gland
- T3b is a new category for tumours of any size with gross extra-thyroidal extension into strap muscles

1.5 Diagnosis and management

1.5.1 Contemporary guidelines

Most recent international best practice guidelines for the management of thyroid cancer come from the American Thyroid Association (ATA, 2015), British Thyroid Association (BTA, 2014) and the European Society for Medical Oncology (ESMO, 2012).[20,52,58,83] In the absence of Irish-specific national guidelines for the management of DTC, BTA guidelines are frequently adopted in Irish centres treating patients with DTC.

Key recommendations from the BTA guidelines include the following[52]:

- Management of DTC should be the responsibility of a multidisciplinary team, normally comprised of a surgeon, endocrinologist and oncologist (or nuclear medicine physician) with support from a pathologist, medical physicist, biochemist, radiologist and specialist nurse, all with expertise and interest in the management of thyroid cancers.
- Use of the TNM staging classification (Table 3), the ATA post-operative risk stratification for risk of recurrence and the Dynamic Risk Stratification should be utilised for prognostication and risk stratification.
- When the evidence for or against a treatment is inconclusive a personalised approach to decision making is advocated.
- Following initial referral for thyroid nodules, ultrasound ± diagnostic FNA should be an initial diagnostic modality of choice. The U1-U5 scoring system should be used for objective ultrasound assessment; patients with U3-U5 nodules should proceed to FNAC. FNA cytology results should be scored by a cytopathologist experienced in thyroid FNAC using the Thy numerical category as defined by the RCPath.
- For patients with Thy3f or Thy4 FNAC results a diagnostic hemithyroidectomy is recommended. Therapeutic surgery is indicated for nodules with Thy5 FNAC results.
- Total thyroidectomy is recommended for patients with tumours > 4 cm in diameter, or of any size in association with any of the following characteristics: multifocal disease, bilateral disease, extra-thyroidal spread (pT3 and pT4a), familial disease, and those with clinically or radiologically involved nodes and / or distant metastases.
- The evidence for an advantage of total thyroidectomy compared to hemithyroidectomy in patients with unifocal tumours >1–≤4 cm in diameter, age <45 years, with no extra-thyroidal spread, no familial disease, no evidence of lymph node involvement, no angioinvasion and no distant metastases, is unclear. Personalised Decision Making is recommended.
- Thyroid lobectomy is recommended for patients with a unifocal microPTC and no other risk factors.
- Patients with tumours measuring >4 cm, or of any size with gross extra-thyroidal extension (pT4), or distant metastases should be recommended for RRA (Figure 8).

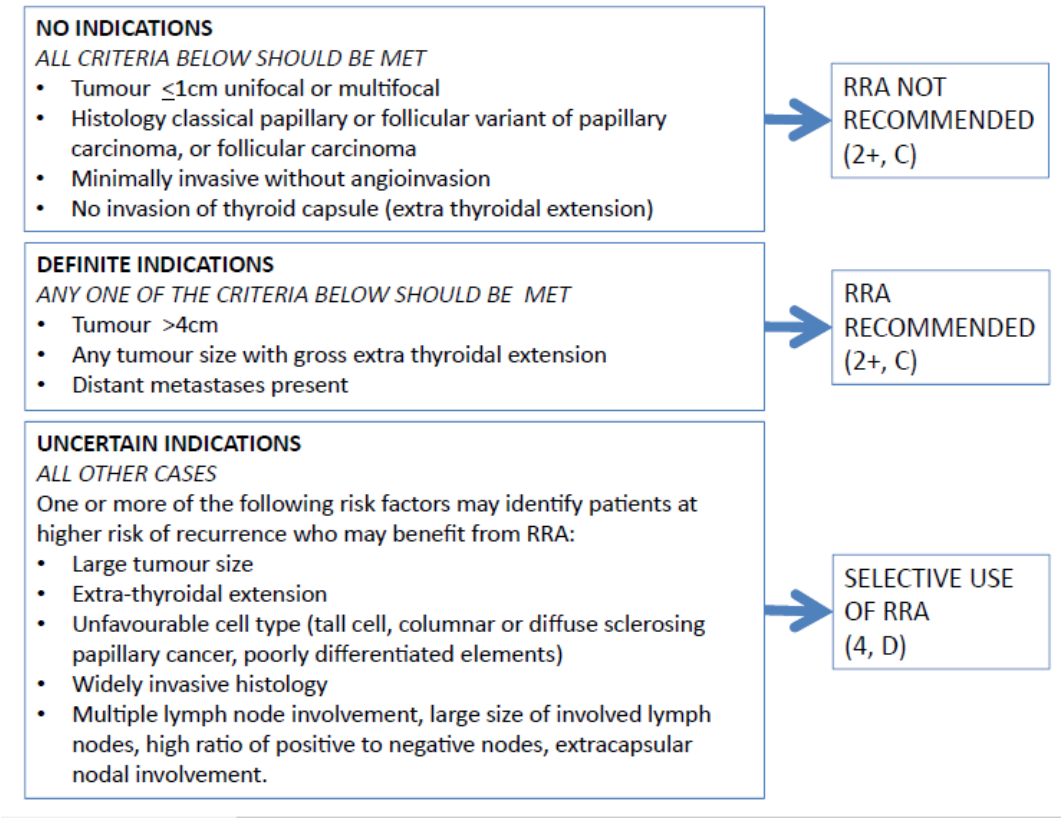


Figure 8: British Thyroid Association indications for RRA (Image reproduced from Perros, BTA Guidelines)[52]

Increased recognition of patient and disease prognosticators in recent BTA and ATA guidelines has facilitated improved risk stratification such that low risk patients, even with tumours > 1cm, may be treated with a more selective and individualised approach while maintaining improved outcomes and reducing treatment related morbidity.[84-87] The 2009 American Thyroid Association (ATA) and 2006 European Thyroid Cancer Consensus guidelines both previously recommended total thyroidectomy for all DTC >1 cm, while more recent 2014 BTA and 2015 ATA guidelines now suggest that lobectomy alone is an option for those with tumours 1 - 4cm without risk factors.[88] Other more conservative measures noted in the 2015 ATA guidelines compared to the 2009 ATA guidelines include a higher threshold for initial FNAC which takes into account both nodule size and radiology U-score, consideration for lobectomy rather than total thyroidectomy in selected cases, reduced indications for RRA in addition to lower I^{131} ablation doses in low-risk DTCs and consideration for active surveillance rather than immediate surgery for selected patients with DTC rather than surgery.[29,58,83]

An overall tendency towards improved risk stratification and a more reserved diagnostic and therapeutic approach represents acknowledgement of the risk of over-treatment given the rapidly increasing diagnosis rates of DTC despite maintenance of globally stable mortality rate in recent years.[89-92]

1.5.2 DTC challenges

The clinical challenge in the diagnosis and management of DTC is to prevent over-diagnosis and over-treatment of those patients with low-risk disease, while also recognising patients with advanced or aggressive disease that require prompt diagnosis and early effective intervention. A number of specific areas in this process pose a challenge to clinician managing DTC and are discussed in the following sub-sections.

1.5.2.1 FNA indications

Formulation of indications for appropriate use of FNA of thyroid nodules poses a challenge for clinicians. Thyroid nodules are common, occurring in up to two-thirds of healthy adults.[93,94] Due to the relative rarity of thyroid cancer compared to the high incidence of nodules, and in the setting of increasing diagnostic imaging modalities and consequent thyroid incidentalomas, over-use of FNA may increase patient morbidity and represent a significant burden on healthcare services, with minimal diagnostic yield. Furthermore, up to 30% of FNACs may be non-diagnostic requiring repeat FNA or potentially unnecessary thyroid lobectomy to establish a diagnosis.[95] Retrospective review of 8,806 patients in California demonstrated that only 1.6% of patients who had 1 or more thyroid nodules 5 mm or greater harboured a thyroid cancer following FNA or surgery.[95]

BTA guidelines recommend FNA for nodules which appear equivocal, indeterminate or suspicious of malignancy on ultrasound (U3-5). They also recommend repeat FNA for nodules returning Thy1 results, or Thy2 cytology but with indeterminate or suspicious US features. There is mixed evidence regarding the utility of nodule size as a predictor for malignancy and as a result BTA do not recommend using nodule size as an index for decisions regarding whether to perform FNA.[52,96,97] In contrast, updated ATA guidelines now suggest a combination of ultrasound scoring and nodule size in an effort to better identify the large volume of low risk nodules; they recommend nodules with a very low suspicion of malignancy require a size of >2 cm to necessitate FNA, nodule with a low index of suspicion must be > 1.5 cm for FNA and those with intermediate or high risk of suspicion for malignancy should be >1cm for FNA to be indicated.

1.5.2.2 Indeterminate FNA

Definitive diagnosis is obtained via FNAC of thyroid nodules in up to three quarters of cases. However, the remaining patients with indeterminate FNAC results often require diagnostic thyroid lobectomy to achieve a definitive diagnosis.[98] Improving this rate of definitive diagnosis without the need for diagnostic surgery is a challenge for endocrine physicians and surgeons, and has been a significant focus of thyroid cancer research efforts in recent years. The incidence of cancer following diagnostic surgery in patients with indeterminate Thy3 nodules is reported by the Royal College of Pathologists and others as 5-15% for Thy3a and 15-30% for Thy-3f FNAC.[99-101] As a result, lobectomy frequently results in diagnosis of a benign lesion on post-operative histological assessment while in cases of confirmed carcinoma, lobectomy alone may prove insufficient as therapeutic surgery requiring subsequent completion thyroidectomy.

Identification of specific biomarkers associated with thyroid carcinogenesis promises to improve the accuracy of thyroid nodule FNAC assessment to reduce the cohort of patients with indeterminate lesions. In the mid 2000's immunohistochemical analysis of Galectin-3-expression in FNA samples showed positive results in improving diagnostic accuracy.[102] Subsequent detection of other specific single-gene mutational markers and rearrangements such as HRAS, NRAS, KRAS, BRAF and Pax8-PPARγ have further shown improvements in the utility of FNA analysis.[103] The Afirma gene expression classifier (GEC) was specifically developed in an effort to decrease the volume of diagnostic lobectomies in patients with indeterminate thyroid nodules, however unfortunately following introduction of its use, there was no significant difference in the volume of surgeries undertaken for patients with indeterminate cytology following FNA and the incidence of nodules classified as indeterminate was seen to increase significantly following its introduction.[104] Multiple other cytomolecular testing tools have since been developed, each with varying diagnostic accuracy, sensitivity and specificity for pre-operative DTC evaluation. These include ThyGenX/ThyraMiR, Thyroseq, Seven-Gene Mutation Panel and the Rosetta GX Reveal tests.[105] Although many of these cytomolecular testing platforms will likely better inform management decisions in the setting of indeterminate cytology, a confident understanding of the advantages and disadvantages of each test is a necessity to ensure they are used appropriately. Furthermore, longitudinal outcome data is not yet available to support their adoption into the standard of care for DTC.

A recent European Thyroid Association expert panel review concluded that molecular testing of FNAC samples may help clinicians to drive patient care and surgical decision making if the analysis is performed in specialised laboratories, however, they include a number of recommendations and caveats with reference to particular established tests and methodologies currently in use.[106] For assessment of cytologically indeterminate nodules, all molecular assessment tools should include

detection of BRAF mutations and RET/PTC rearrangements, and, possibly, PAX8/PPARG rearrangements and RAS mutations. In particular, they specify that the role of RAS mutation detection needs further clarification. They advise that the Afirma GEC should not currently be routinely used for patients with indeterminate FNAC given the absence of long-term outcome data and debate in the literature regarding its associated positive and negative prediction values. They also caution that although the use of targeted next-generation sequencing (tNGS) methods are promising, their use should be limited to research settings until issues regarding standardisation of data analysis, mutation cut-offs and standard operating procedures are addressed in multiple large laboratories. As a general guide, the European Thyroid Association expert panel agree with the National Comprehensive Cancer Network guidelines that all molecular tests should aim for an ideal PPV >95% for rule-in FNAC analysis which would allow for up-front surgical planning of more radical resection where indicated thereby avoiding 2-stage treatment, while FNAC molecular assessment strategies aiming to rule-out cancer should have an ideal NPV >95% in order to avoid surgery.[106,107]

1.5.2.3 Extent of surgery

Total thyroidectomy is advised by the BTA for patients with Thy 5 FNAC or with confirmed DTC following diagnostic lobectomy where tumour size exceeds 4 cm, or measures any size in association with specific risk factors including multifocal disease, bilateral disease, extra-thyroidal spread (pT3 and pT4a), familial disease, and those with clinically or radiologically involved nodes and / or distant metastases.[52] Lobectomy alone is deemed sufficient for patients with unifocal papillary microcarcinoma (microPTC, <1 cm) without risk factors; these include multifocality, larger size (6-10mm), extra-thyroidal extension, poor differentiation and a desmoplastic fibrosis or an infiltrative growth pattern.

However, there is a dearth of well designed, peer-reviewed randomised or prospective studies to support an advantage of total thyroidectomy over lobectomy in patients with unifocal tumours measuring 1-4 cm in diameter, age <45 years, and without extra-thyroidal spread, familial disease, evidence of lymph node involvement, angioinvasion or distant metastases. In these cases, BTA guidelines recommend a "Personalised Decision Making" approach which advocates a shared doctor-patient decision making process in conjunction with multi-disciplinary team (MDT) input, with due consideration for recurrence risk, patient comorbidities, and personal circumstances and values.[52]

This clinical scenario represents a difficulty for patients and the multi-disciplinary team who must assess a variety of patient, disease and physician factors to choose the most appropriate treatment

plan on an individualised basis. Recently updated risk stratification methods and updated staging classification systems provide improved guidance in the short term, however, molecular medicine may provide key prognosticators and risk indicators to further reduce the volume of patients falling into this grey area of 'personalised decision making'.

1.6 Thyroid cancer genetics and molecular biology

Our understanding of the molecular and genetic characteristics of thyroid cancer has evolved significantly over the past decade. The overall prognosis and survival rates associated with DTC are high compared with many other malignancies and this is reflected in its relatively low mutation frequency as determined by whole-exome sequencing.[108] In a similar fashion to many other cancers, it is postulated that the degree of invasiveness, grade and de-differentiation associated with tumours originating from follicular epithelium is associated with an accumulation of mutations such that tumours may progress from benign adenomas to DTC and onward to poorly differentiated or anaplastic carcinomas (Figure 9). Increasing numbers of point mutations and rearrangements within a given tumour are associated with a poor prognosis and this is acknowledged in the 2015 American Thyroid Association Guidelines for the management of thyroid cancer (Figure 10).[58]

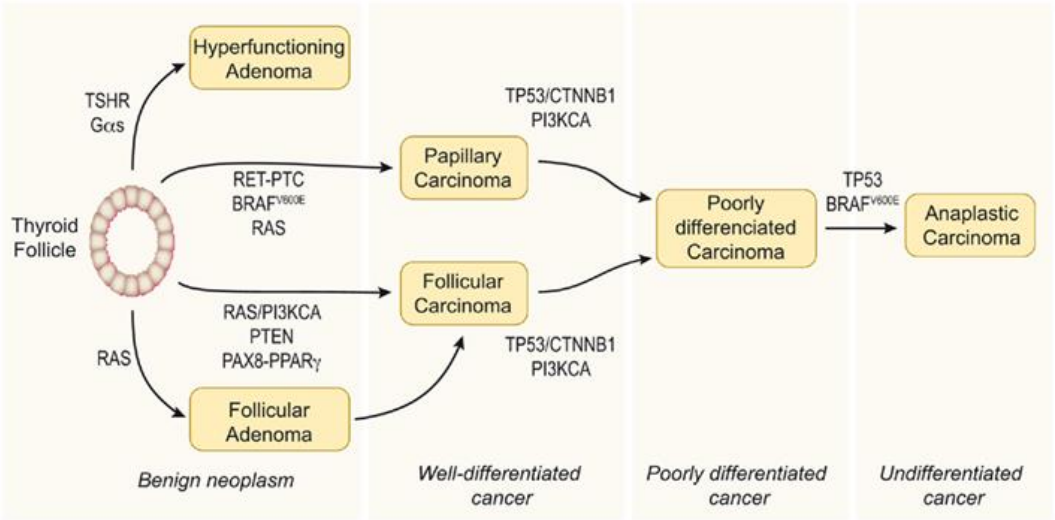


Figure 9: Stepwise model of thyroid carcinogenesis (Image reproduced from Bychkov)[109]

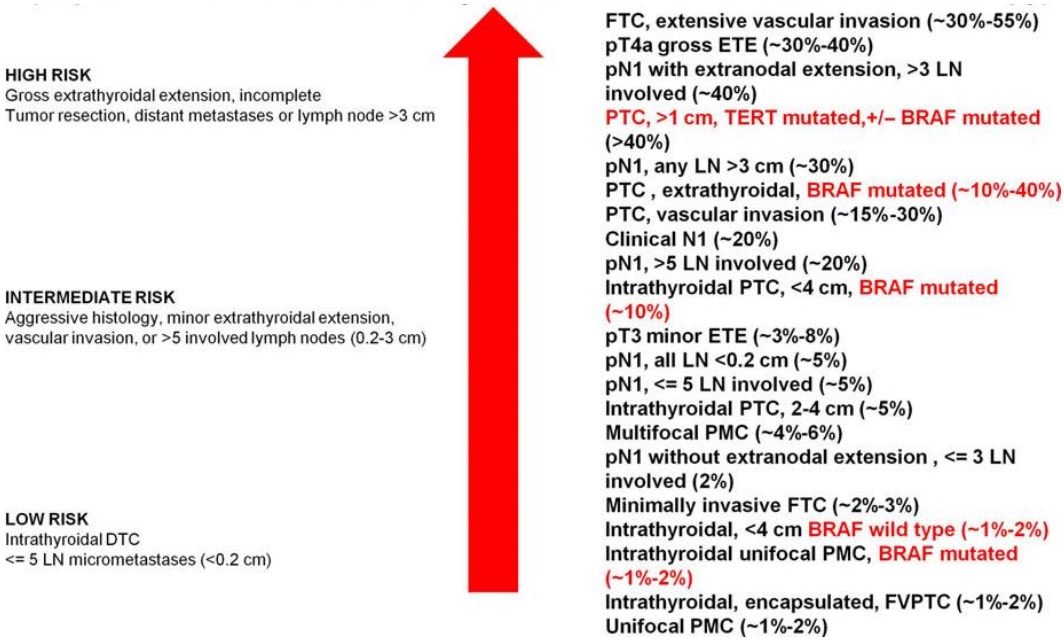


Figure 10: Risk of structural disease recurrence in patients without structurally identifiable disease after initial therapy (Image reproduced from Haugen, ATA Guidelines 2016)[58]

PTCs demonstrate mutations in the mitogen-activated protein kinase (MAPK) pathway (Figure 11). The most common of these include BRAF V600E mutations in up to 60% of PTCs, RAS mutations in 15% of PTCs and RET/PTC, ALK or NTRK1 chromosomal rearrangements in 12% of papillary carcinomas.[108,110] Other implicated co-existing mutations include TERT promoter, PIK3CA, AKT1 and TP53 mutations. Discrete driver mutations have not yet been elucidated for a number of PTCs that demonstrate none of these mutations or rearrangements. In many cases, presence of a specific driver mutation may confer distinct clinical and histological PTC phenotypes and behaviours; BRAF V600E mutant tumours exhibit high rates of lymph-node metastases, poor response to radioiodine treatment and high rates of recurrence after thyroidectomy. BRAF V600E mutations are present in 90% of tall-cell PTCs compared to 60% of classical PTCs.[108]

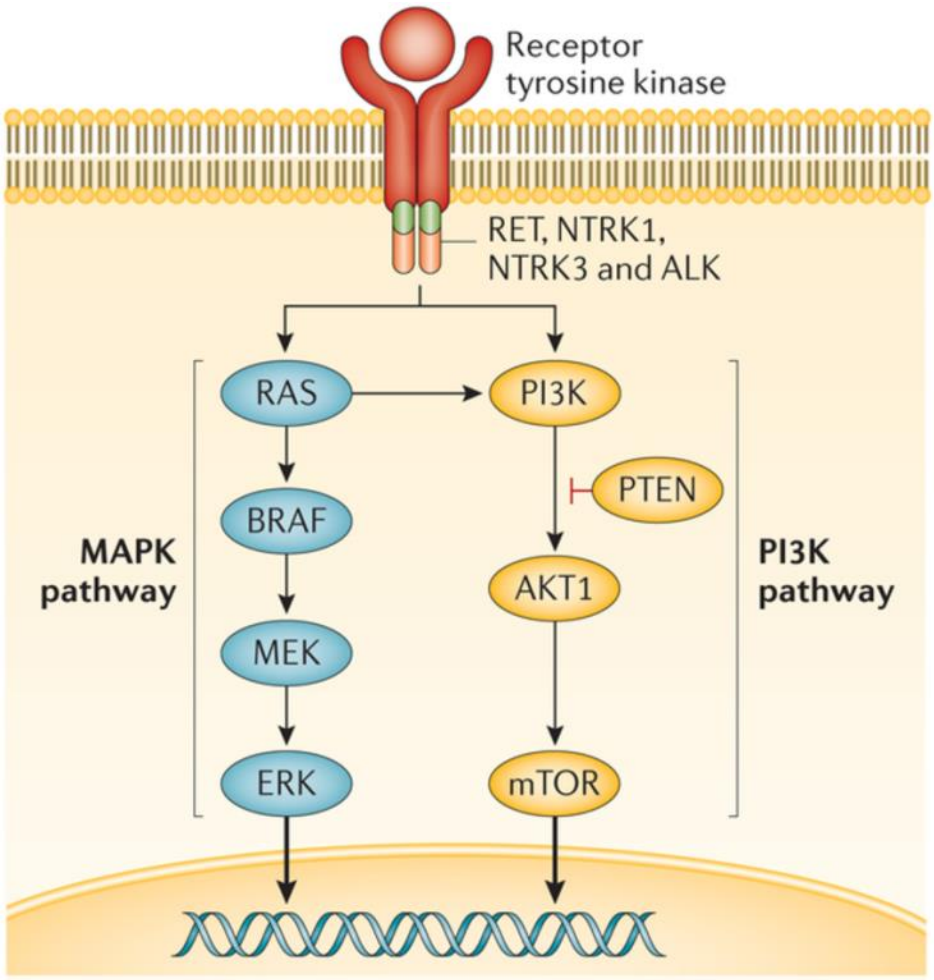


Figure 11: Mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways which become chronically activated in follicular cell-derived thyroid carcinogenesis and progression (Image reproduced from Dralle)[111]

RAS mutations are typical of follicular neoplasia and are recognised in over 50% of FTCs, follicular adenomas and fvPTCs.[112] FTCs exhibit mutations mainly in NRAS, but also in HRAS and KRAS, in association with the presence of mutually exclusive PAX8/PPARγ rearrangements. [112,113] Less frequently, FTCs harbour mutations in BRAF K601E and EIF1AX, and have RET/PTC-3 rearrangements.

Given the overall predominance of BRAF mutations in PTC, and RAS mutations in FTC, these tumour groups have been described as BRAF-like and RAS-like respectively and provide another classification system for thyroid tumours, which although is consistent with current histological architectural divisions, does not correlate well with cytological classifications, as evidenced by the inclusion of fvPTC in the RAS-like group.[112]

While the focus of much genetic mutation analysis has been on somatic MAPK and PI3K pathway mutations detected in FNAC samples and thyroid tissue, there is likely a role for further delineation of risk based on germline mutational analysis, particularly given the significant heritability of DTC.[114,115] These may include novel variants identified by genome-wide association and candidate-gene studies or germline mutations in genes that are already known to undergo somatic mutations in thyroid and other tumours.

1.7 Single nucleotide polymorphisms

The human genome comprises the complete set of nucleic acids which make up the DNA within the 23 chromosome pairs found in the nucleus of every human cell (Figure 12). In 2003, the Human Genome Project completed the 15-year-long sequencing of all three billion base pairs of the human genome.[116,117] There is an abundance of variation within the human genome, with multiple variants of any given gene evident between humans; this is termed polymorphism. This genomic variation manifests as the unique phenotype of every person, but also accounts for disease and illness. Less than 2% of the human genome can be transcribed into messenger(m)RNA and translated into proteins; there may be as few as 19,000 human protein-coding genes.[118] The remaining 98% of our genome is classed as non-coding and although not fully elucidated, may be involved in functions such as regulation of gene expression, organisation of chromosomal architecture or control of epigenetic inheritance. [118]

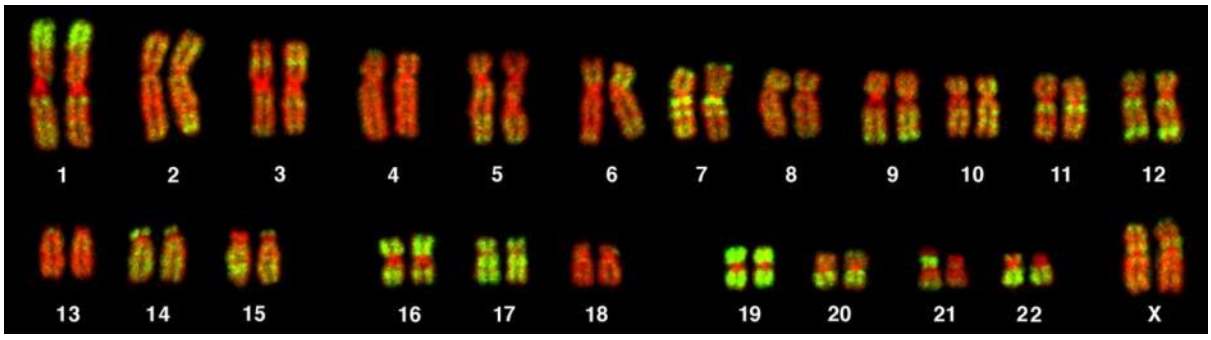


Figure 12: Karyotype from a female human lymphocyte (46, XX) with DNA visualised using fluorescence in-situ hybridisation (Image reproduced from Bolzer)[119]

There are numerous mechanisms through which genomic variation comes about, ranging from large scale chromosomal number and structure variations such as aneuploidy and polyploidy, which are

visible under light microscopy down to single nucleotide substitutions along a chromosome, termed single nucleotide polymorphisms (SNPs) (Figure 13).

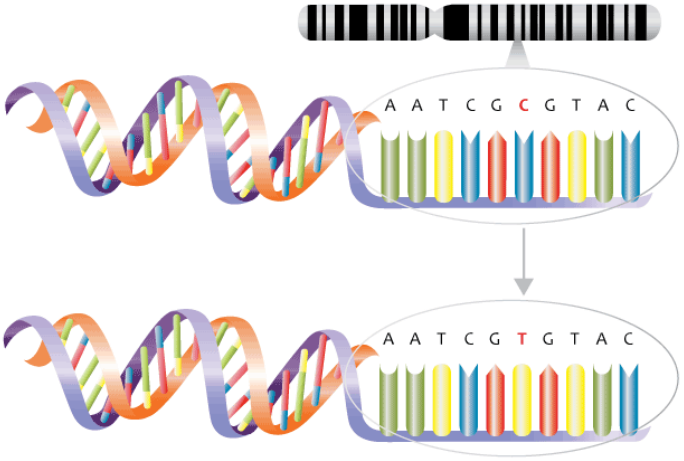


Figure 13: Single nucleotide polymorphism. Note variation in DNA at a single base-pair (Image reproduced from NHS)[120]

SNPs represent the most common type of genetic heterogeneity, estimated to account for 98% of genetic variation and occurring approximately once in every several hundred base pairs, although with a non-uniform density throughout the genome. They occur more frequently in non-coding regions than in DNA coding regions, although SNPs in non-coding introns may still affect functional proteins by altering transcription factor binding, gene splicing or mRNA degradation. The 1000 Genomes Project attempted to catalogue all genetic variants with frequencies of at least 1% in the populations studied. Following reconstruction of genomes from 2,504 individuals from 26 populations, they identified over 84 million SNPs.[121] It is estimated that any given person carries between 2.8 and 3.9 million single-base pair variants.[122] Another international consortium known as the HapMap Project was established in 2002 with a goal to identify these variants in the human genome, and to examine their distribution among various world populations. This data is freely available for use in ongoing genetic research.[123]

Given that humans are diploid organisms, with one allele inherited from each parent, we each have two alleles at any given genetic locus. Each pair of alleles constitutes the genotype of a given gene, which are described as homozygous if the genes are the same on each allele and heterozygous if they differ. As a result, we have the potential to have a SNP in none, either or both alleles, resulting in three potential genotypes for a given SNP (Figure 14). With respect to any particular SNP, a person

may be a heterozygous variant carrier if the SNP is present on either allele only, a rare homozygous carrier if the SNP is present on both alleles or a common homozygous carrier if the SNP is absent from both alleles (also known as the wild type genotype). SNPs may be located in any portion of a gene, including promoter regions, exons, introns as well within 5' and 3' untranslated regions (UTRs). Depending on their location, and the function of each gene, SNPs may have genetic or epigenetic mechanisms that give rise to cancer susceptibility; it follows that validated SNPs may be utilised as predictive cancer biomarkers.[124]

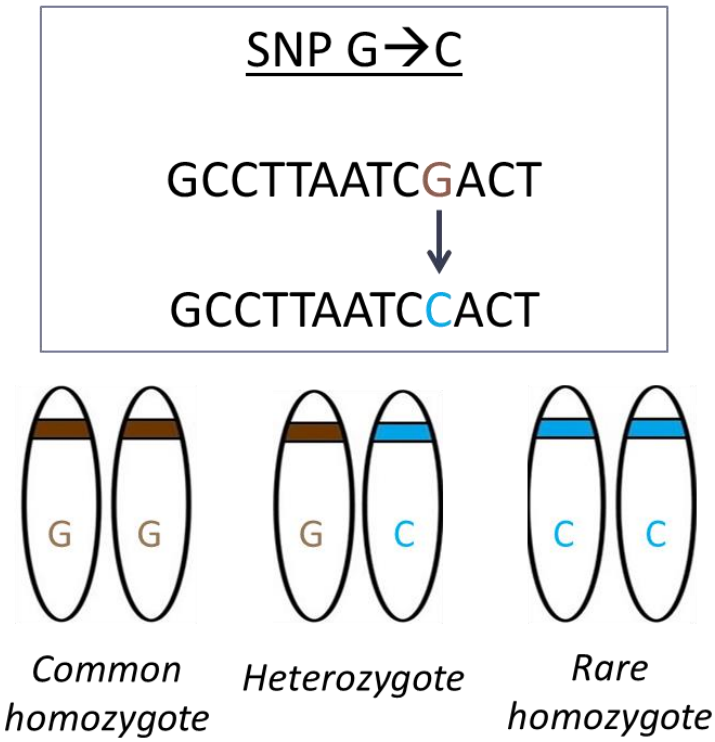


Figure 14: Potential genotypes for a guanine → cytosine SNP at any given human locus

1.7.1 Genome-wide association studies

Following completion of the Human Genome and HapMap Projects, researchers worldwide had access to complete datasets detailing the whole human genome, which could be used as a reference and facilitate a novel method of genetic study known as a genome-wide association study (GWAS). GWASs are a type of observational, case-control study whereby the distribution of genetic variants is assessed across the whole genome of many participants with a known phenotype (DTC for example) and compared with the distribution of variants in a control group without that phenotype. This allows researchers to assess whether any particular variants consistently appear more frequently in the affected cohort compared to the control group. Many GWASs focus on associations between

SNPs and disease phenotype. GWASs are known as non-candidate-driven studies given that they assess the entire genome. This type of study was made possible not only by the development of the HAPMAP and similar projects but also as a result of improved software and technology such as DNA microarrays which facilitate rapid genotyping of large amounts of DNA. [125,126] Although GWASs may establish a link between particular SNPs and associated illnesses, they do not explain the mechanisms through which any given SNP may lead to a particular disease and the identification of a SNP associated with a particular phenotype does not infer causation. Many illnesses have a multifactorial aetiology which may include germline and somatic mutations in addition to complex environmental factors. Therefore, GWASs are not absolute predictors of disease but may serve to inform our knowledge of disease susceptibility and risk.[126]

1.7.2 SNPs and GWAS in thyroid cancer

Inherited genetic predisposition is believed to be a significant aetiological factor in the development of non-medullary thyroid cancer. In the absence of significant thyroid cancer-specific high-risk, high penetrance gene mutations, such as the RET mutation associated with MTC or PTEN mutation associated with Cowden's syndrome, it is postulated that multiple low to medium risk sporadic mutations may occur simultaneously in any given person, thereby exerting a polygenic effect and increasing susceptibility to DTC (Figure 15).[127]

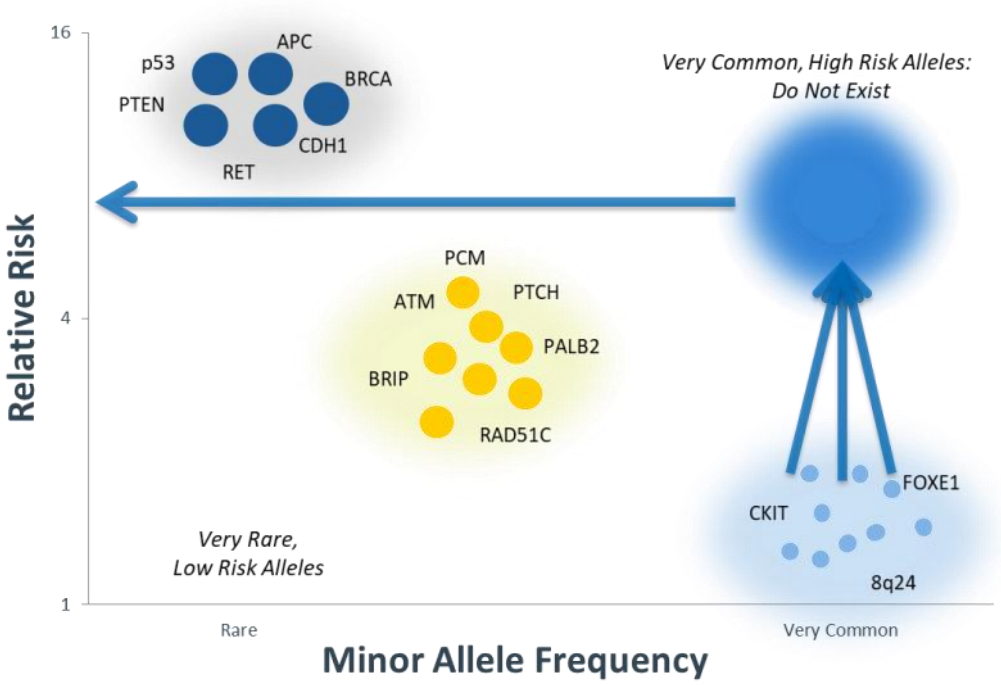


Figure 15: The polygenic effect of multiple low risk alleles cumulatively increasing DTC risk

GWASs represent an important tool to facilitate identification of these low-risk mutations which may increase DTC susceptibility. A number of GWASs have been undertaken to date which have identified SNPs that confer increased risk for development of DTC. The first ever reported GWAS for thyroid cancer was performed by Gudmundsson et al in 2009, who assessed 192 Icelandic patients with differentiated thyroid cancer and 37,196 controls. They identified two SNPs, rs965513 on 9q22.33 near the FOXE1 gene and rs944289 on 14q13.3 near the NKX2-1 gene with observed odds ratios of 1.75 ($p=1.7\times10^{-27}$) and 1.37 ($p=2.0\times10^{-9}$) respectively for DTC. FOXE1 and NKX2-1 are also known as thyroid transcription factor genes 1 and 2. This odds ratio rose to 5.7 for homozygous carriers of both SNPs which occurred in 3.7% of their DTC cases. Another early GWAS published in 2010 by Takahashi et al also reported on Rs965513 in 172 controls and 187 patients with radiation-related PTC following a history of exposure in the Chernobyl nuclear accident. They examined for 532,024 SNPs and reported that rs965513 conferred an associated odds ratio of 1.65 (95% CI: 1.43–1.91).[128] Gudmundsson's group further reported findings in 2017 from a genome-wide association study of 3,001 non-medullary thyroid cancers and 287,550 controls, yielding five novel SNPs with odds ratios ranging from 1.32 to 1.81 ($p<3\times10^{-8}$ for all).[129] Further GWASs in thyroid cancer have been undertaken by Ho-Young Son et al, Kohler et al, Figlioli et al. Mancikova et al, Wang et al, Matsuse et al, Liyanarachchi et al and Jones et al [130-139] A number of SNPs have been

identified, some of which have been replicated in multiple GWASs. These include SNPs located near FOXE1, NKX2-1, DIRC3, NRG1, IMMP2L, RARRES1, SNAPC4, BATF, DHX35, GALNTL4, HTR1B and FOXA2 genes. The rs965513 FOXE1 SNP is the most robustly reported SNP with an odds ratio (OR) of 1.80 in Caucasians reported in one meta-analysis.[130,140]

1.7.3 Candidate gene studies in thyroid cancer

GWASs test an entire genome for genotype–phenotype associations and therefore do not require knowledge of at-risk genes prior to undertaking of the study. This facilitates discovery of new gene mutations that may not have previously been known. However, due to the large number of independent tests performed throughout a GWAS, they tend to be underpowered and may result in significant numbers of false positives, even in the presence of large sample sizes with thousands of subjects. Apart from sample size affecting statistical power, mutations with small, but significant, effects are difficult to discover by GWAS due to the low power of the analysis.

The candidate gene approach is another commonly utilised form of genotype–phenotype study whereby a group of patients with a particular disease and a group of healthy controls are assessed for the presence of a given mutation or SNP to determine whether one allele of a candidate gene is more often seen in those with disease than in those without. The main advantage of candidate gene studies is that they generally confer a higher statistical power that GWASs, however given that they require knowledge of the SNP being assessed prior to the study, they are not capable of discovering novel genetic mutations.[141,142] Although knowledge of the target mutation prior to a candidate gene study may inherently introduce bias in contrast to GWASs, the value of the candidate gene approach lies in the ability to detect increased risk or susceptibility even where the minor allele frequency is low.[141,143] This is particularly relevant given that DTC phenotype may be determined by many gene mutations of small effect size. Furthermore, following initial identification of a mutation by GWAS, the candidate gene approach enables researchers to quantify the extent of variability in effect size with geographical or environmental factors across various populations.[144] The population of Ireland is favourable for the study of genetic variability given its relatively reduced genetic diversity compared to many other continental European nations. The relatively homogenous population genetics of the Irish people is attributable to their island location, geographically isolated from continental Europe, historically low rates of inward migration and the relatively late human colonisation of Ireland compared to the rest of Europe.[145] Conversely, given traditionally high rates of emigration from Ireland, results from Irish gene mutation studies are relevant internationally.[145,146]

1.8 MiRNAs

Micro RNAs (miRNAs) are short non-coding RNAs, measuring 21-24 nucleotides long, that exert post-transcriptional effects on gene expression. They were initially discovered in 1993 by Lee, Ambrose and colleagues while examining the lin-4 gene and its effect on *Caenorhabditis elegans* larva development. Following isolation of the lin-4 gene, they noted that this gene produced a short non-coding RNA rather than mRNA encoding a protein, as they had expected.[147] Subsequently, Reinhart et al demonstrated another gene, let-7, which encoded another short RNA measuring 22 nucleotides long. This RNA was found to promote progression from larval to adult cells, again, in *C. elegans.*[148] Subsequently in the early 2000's a plethora of similar genes encoding short regulator RNA strands were elucidated, and thus miRNAs were found to encompass a broad class of small regulator RNAs evident throughout many animal and plant species, with multiple miRNAs phylogenetically conserved.[149] miRNAs have since been determined to be important regulators of gene expression and function in the genome of many eukaryotic species, including humans. Currently, 38,589 precursors and 48,860 mature microRNAs are recorded in MiRBase, a public biological repository established in 2002 which archives, names and distributes microRNA gene sequences. Typically miRNAs interact with specific mRNAs through complementary base-pairing to influence the translation or stability of the target mRNA molecule, however, they may act at many other important levels of the genome including chromatin structure, chromosome segregation, transcription, RNA processing and RNA stability.[150]

MiRNAs act as gene regulators in animals, typically by binding to complimentary sites in the 3' untranslated region (UTR) of mRNA, forming a complex known as a miRNA-induced silencing complex (miRISC), which results in silencing or inhibition of protein translation from the target mRNA.[151] MiRNAs have been shown to be involved in almost all aspects of normal human biology including stem cell differentiation, haematopoiesis, apoptosis, cardiac and skeletal muscle development, neurogenesis, insulin secretion, cholesterol metabolism and the immune response.[151]

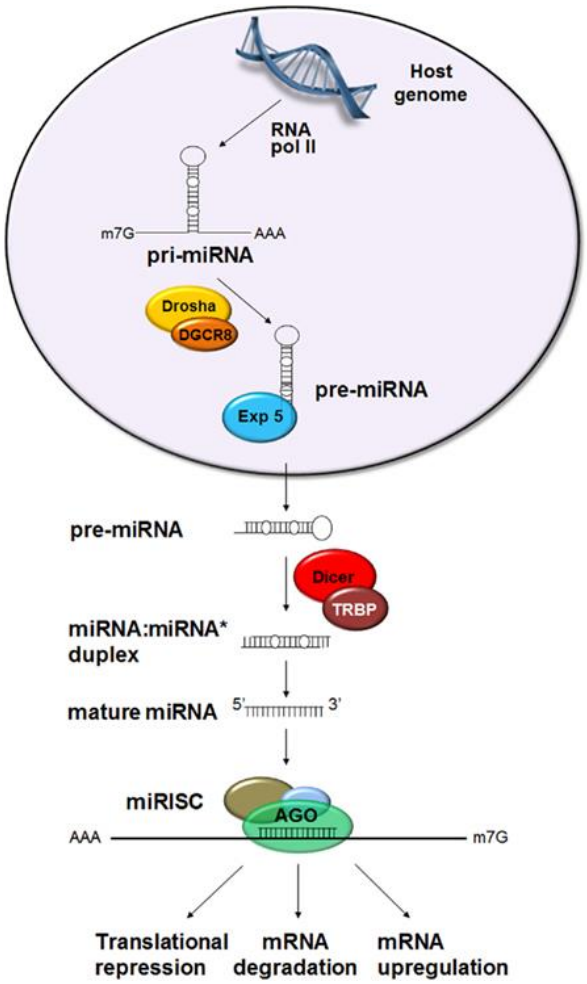


Figure 16: MiRNA biogenesis & function. RNA polymerase II expresses miRNA genes forming primary miRNA (pri-miRNA), which is then cleaved by Drosha to form precursor miRNA (pre-miRNA), which is exported out of the nucleus. Following cleavage by Dicer, a miRNA:miRNA duplex is formed with the passenger strand usually being degraded, leaving a mature single strand miRNA. This miRNA then attaches to the 3'UTR of mRNA to form a miRNA-induced silencing complex (miRISC) resulting in repression of translation, mRNA degradation, or mRNA up-regulation (Image reproduced from Asgari)[152]

While miRNA play an integral role in normal cell function, dysregulation of miRNA may be associated with disease. MiRNAs have been demonstrated to regulate cell differentiation and proliferation; failure of these processes to be regulated may result in carcinogenesis, with many cancerous tissues expressing miRNAs at a lower level than in equivalent healthy tissue.[151] Reduction in these miRNAs suggests a tumour suppressor role. Similarly, up-regulation of various miRNAs has also been described, with these miRNA being described as oncomiRs. The first disease state to be associated with miRNA in humans was chronic lymphocytic leukaemia.[153] Many cancers have subsequently been linked with miRNA function and dysfunction, including differentiated thyroid cancer.[154,155]

MiRNA function can be dysregulated secondary to the occurrence of single nucleotide polymorphisms. Given the mechanism of miRNA/mRNA interaction, miRNA function may be affected

by SNP occurrence in the seed miRNA gene sequence (miR-SNP) or within a 3' UTR target site of the miRNA (miR-TS-SNP), both with the potential to affect the expression of microRNA targets.[156] Furthermore, increasing numbers of SNPs have been reported in various stages of miRNA biogenesis, including pri-, pre- and mature miRNA sequences (Figure 16).[156] Given that miRNA can have hundreds of binding sites, it follows that miR-SNPs have the potential for more significant biological effects than miR-TS-SNPs.[157] MiRNA sequences are very short and have been shown to be relatively well conserved, which contrasts with the large numbers of less conserved non-coding 3' UTRs in the human genome, indicating that the frequency of binding site SNPs is much higher that miR-SNPs, with each miR-TS-SNP exhibiting more discrete effects compared to miR-SNPs, which may be more desirable for genotype-phenotype case-control studies.[158]

1.8.1 MiRNAs and thyroid cancer

MiRNA dysregulation has been implicated in DTC carcinogenesis.[159] Several studies have assessed miRNA in DTC with a subset being commonly dysregulated in a number of studies in varying populations; these include miR-146a, miR-146b, miR-222, miR-221 and miR-181b.[27,159,160] MiRNA expression profiles and measurement of circulating miRNA may prove to be of significant utility for the diagnosis and prognostication of patients with DTC.

Numerous studies have examined the utility of miRNA expression profiling in serum and diagnostic FNA samples in the setting of indeterminate cytology results. Pallante et al reported fine-needle aspirates with between 5 and 35 fold over-expression of miR-221, miR-221 and miR-181b in 30 patients with PTC while miR-100, miR-125b, miR-138 and miR-768 showed >5-fold expression difference between benign and malignant thyroid neoplasms on miRNA array analysis of aspirates in a study by Vriens and colleagues.[161,162] A recent systematic review summarised that expression levels of miRs-21, −34b, −130b, −135b, −146b, −151, −181b, −199b-5p, −221, −222, −451, −623, −1271, −2861, and let-7e were each significantly associated with at least one aggressive clinico-pathological thyroid tumour feature.[163] Most studies to date examining the impact of miRNA expression profiles on disease phenotype have been retrospective. Well-designed prospective studies are necessary prior to recommending miRNA profiling as part of DTC management pathways.

1.9 Conclusion

Differentiated thyroid cancer is an increasingly heterogeneous disease with a wide spectrum of phenotypes ranging from indolent to aggressive variants. Increasing incidence in recent decades has partly arisen due to intensified surveillance and resulted in an increasing incidence of early cancers, many with indolent behaviour patterns, although there also has been a notable concurrent upsurge in aggressive PTC phenotypes.[15,164] Current best practice guidelines for diagnosis and management do not adequately delineate between these divergent risk subgroups, owing to a paucity of good quality evidence to support recommendations for risk stratification and optimal therapies. The key to improving risk stratification and prognostication for patients with DTC may lie in an improved understanding of the molecular mechanisms of thyroid tumourigenesis, and knowledge of low risk mutant alleles which contribute to DTC susceptibility. Although the pathological mechanisms of thyroid cancer susceptibility are not yet fully understood, with recent advances in technology, it is unlikely that a significant high penetrance monogenic variant, such the RET proto-oncogene for MTC, remains undiscovered for DTC, however, detection of low penetrance alleles across multiple genes, while conferring small effect sizes individually, may cumulatively exert an overall polygenic effect approaching effect sizes more often associated with high-risk cancer syndromes.

The hypothesis of this study is that a significant number of Irish patients with DTC are subject to equivocal guidelines and may be over-treated. This work will describe patterns of DTC presentation and management in tertiary cancer centres, and assess the therapeutic approaches in our cohort as compared to current British Thyroid Association clinical practice guidelines. We ascertain the effect of equivocal guidance, particularly for patients with intermediate size 1-4cm tumours, on contemporary practice patterns. Additionally, with a view to improving risk stratification for DTC cohorts subject to equivocal guidelines, we validate the effect of single nucleotide polymorphisms in genes affecting thyroid biology for patients with DTC, and investigate the role of these genotypes in modifying DTC risk. We also examine the impact of mutations in miRNA, or in miRNA-mRNA binding sites on DTC susceptibility. In order to facilitate both clinical and experimental aspects of this work, we initially establish and populate a multicentre thyroid cancer biobank and complementary clinical database, as a tool for examining both genetic and clinico-pathological characteristics of differentiated thyroid cancer.

2 Chapter 2 - Materials and methods

2.1 Biobank

Biobanking is a relatively recent concept in the world of medical science, with widespread publication not evident until approximately 2006 (Figure 17).Biobanks are repositories of biological samples with accompanying linked clinical data and represent a significant resource for researchers allowing access to a huge range of samples and thus facilitating large scale analysis of human tissue.[165] They facilitate collaborative work on a large scale by acting as a resource for many researchers, examining a variety of research questions over a long period.

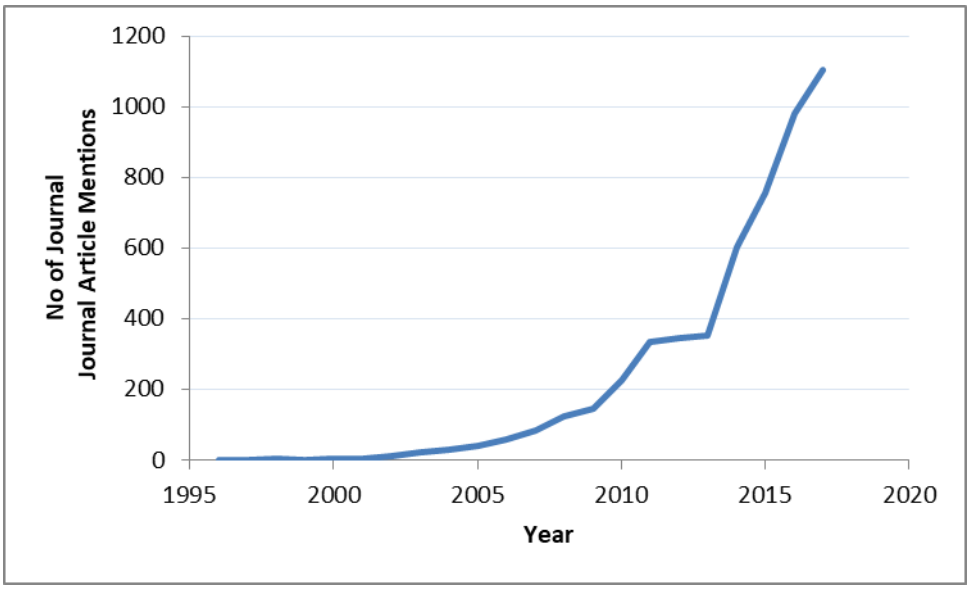


Figure 17: 'Biobank' article mentions in Pubmed indexed journals

A number of key elements and resources required for establishing a Biobank. These include physical resources such as safe secure storage facilities, human resources to obtain samples, book them into the lab system, stabilise tissues for storage and then for processing such as DNA or RNA extraction if required. Specimen types often include whole blood, plasma, urine, cerebrospinal fluid, saliva or tissue samples. Tissue samples then can be from a variety of sites, including normal tissue samples, tumour samples and tumour-associated normal tissue.

Biobanking presents a unique set of ethical and security considerations concerning the long-term collection and storage of human biological materials. While patients and control populations are usually eager to contribute to medical research, some may feel uncomfortable with the concept of

their tissue being stored for possibly many years to come for laboratory research not yet even conceived. As a result, appropriate consent protocols and documentation are crucial to maintaining patient confidence in such research methods, including this work. The US National Bioethics Advisory Commission have suggested that one solution may be for patient consents to be graduated, such that they may choose the level of consent they provide for the use of their samples, ranging from use in a single defined research project up to open-ended consent for future unspecified research.[166] Another potential minefield in the area of biobank ethics concerns ownership of biological samples and resulting gains that may ensue from the use of biological tissues. While the BioBank is responsible for security and storage of the banked tissue, do they really own it? These issues present ethical challenges for clinicians and laboratories worldwide. Existing guidelines are available from a variety of regulatory bodies, in Ireland and abroad, regarding the practical, ethical and legal issues surrounding the storage of human biological materials for research.[166,167] The establishment of such large banks of human tissue is essential for the advancement of biomolecular research.

2.1.1 Existing tissue biobank and SHIRE database

A population genetics research programme has been ongoing at the Discipline of Surgery, National University of Ireland (NUI) Galway since 1992. Integral to the success of this programme has been the establishment of a tissue repository, located in the Lambe Institute for Translational Research, NUI Galway, which houses blood and tissue specimens from patients with cancer, non-malignant disease and also from non-diseased controls. The Discipline of Surgery biobank currently houses samples from over 5,000 patients. Although initially focusing on collection of breast tissue samples to facilitate research in breast cancer genetics, the Discipline of Surgery biobank now includes samples from patients with other malignancies including melanoma, colorectal and urological cancers. Samples stored include whole blood, serum, plasma and fresh frozen tumour and normal tissue. Additionally, extracted nucleic acids from these samples are stored. All biobank data and inventory is managed using the Shire Data Management System. The Shire genetic database system comprises a fully integrated comprehensive patient and tissue database solution enabling cytogenetic, DNA & biochemical sample tracking in a secure environment. This database is prospectively maintained at the Discipline of Surgery NUI Galway, and includes clinico-pathological details in addition to specimen parameters for all enrolled subjects. This Biobank is an invaluable resource for research, and it has been utilised not only by researchers within the department, but also in international consortia, including the London Cancer Research Institute, the Breast Cancer Association Consortium (BCAC). Research collaborators include the UC Davis Genome Center,

University of California, University of Oxford, Yale University, Baylor University, the Mayo Clinic, Cancer Trials Ireland (formerly the All Ireland Co-Operative Oncology Research Group, ICORG) Nottingham Trent University, and various research departments within NUI Galway, including the Regenerative Medicine Institute (REMEDI) and the National Centre for Biomedical Engineering Science (NCBES).

All biological samples and associated clinical data were collected and stored with ethical approval. Specifically, the consent process utilised was explicit, clear and unambiguous as required by GDPR, while all health and genetic data, including all data derived from testing on biological samples, is sufficiently anonymised. Furthermore, data collection practises conformed to the principle of data minimisation such that all collected data was relevant and necessary in relation to the research question.

2.1.2 Establishing a thyroid biobank & ethics

Ethical approval for the establishment of a thyroid cancer biobank, including collection of blood, tissue and clinical data from patients with thyroid disease, was obtained from the Galway University Hospitals Clinical Research Ethics Committee. An amendment was granted to protocol number 45/05 and C.A. 151, 'The provision of a breast cancer biobank research resource for use in molecular and cellular studies and clinical trials' such that sampling would extend to include patients with cancers of thyroid origin (Appendix 8.1).

2.1.3 Consent and patient information

Prior to inviting patients or members of the public to participate in this research, informed written consent to be included in the NUI Galway biobank and clinical database was obtained (Appendix 8.2). All participants were aware that inclusion would involve no clinical risk outside their standard investigation and treatment, all clinical data would be anonymised, and that a decision not to participate, or to withdraw at any time, would not affect their standard of treatment or clinical decision making in any way. All patients were specifically consented for collection of blood samples, tissue samples and collection of clinico-pathological information.

2.1.4 Case data and sample collection

Cases for inclusion in this study included those being investigated and treated for thyroid cancer. Patients were identified at a number of clinical encounters such as while attending for definitive surgical management or attendance at specialist thyroid cancer follow-up clinics. Clinical sites for data collection included hospitals within the Saolta University Healthcare Group hospitals in Ireland and patients attending Hôpital de la Timone, Marseilles, France. As discussed in Section 1.73, the relatively homogenous population genetics of Ireland, in addition to historically low rates of inward migration and high rates of emigration, result in population traits which are desirable for genetic mutation studies. Inclusion of case samples from French patients is also desirable given that the incidence of DTC in France is among the highest in Europe, particularly in the south-east; the thyroid cancer incidence in Départements Isère, is reported by the World Health Organisation as 20.6 per 100,000.[168,169]

Data was collected over a three year period between August 2014 and August 2017. All patients included in the study were discussed at an endocrine multi-disciplinary meeting typically attended by at least one endocrinologist, endocrine surgeon, radiologist, radiation oncologist and pathologist. Two sample collection methods were employed for this study; either a 10 mL sample of EDTA stabilised whole blood taken at the time of phlebotomy otherwise indicated as part of routine management, or via salivary sample collection using DNA Genotek Oragene 575 collection kit. A clinical details questionnaire/proforma was used to record clinico-pathologic data for each patient included in the study, including risk factors, personal and family history of malignancy (Appendix 8.3).

2.1.5 Control data and sample collection

Controls were defined as persons over the age of sixty years without any current or previous diagnosis of cancer (excluding non-melanomatous skin cancer), and without any first degree relative with a current or previous history of thyroid cancer. Existing biobank controls were recruited from non-oncological outpatient clinics in Saolta University Healthcare Group hospitals, and from volunteers in the community from retirement groups and sports clubs. Although a large cohort of controls already existed in the biobank prior to the commencement of this work, the majority were female patients previously recruited as controls for breast cancer research. To further increase the number of available non-cancer controls, in particular male participants, a recruitment drive was undertaken at a national 'Active Retirement Ireland' event. All controls who donated a tissue sample

by way of buccal swab or blood sample also completed a clinical details questionnaire (Appendix 8.4).

2.2 DNA extraction

The processes employed for extraction and purification of DNA from DNA Genotek ORAcollect OCR-100 specimens, and from whole blood specimens, are each detailed below.

2.2.1 Genotek ORAcollect-buccal DNA swabs

DNA Genotek ORAcollect OCR-100 buccal swabs are a fast, non-invasive method of obtaining reliable DNA samples for molecular applications, providing a 1mL liquid sample with a median DNA yield of 3.9 µg, which is stable at room temperature for at least a period of months (Figure 18, Appendix 8.5).[170] Although they do not provide a DNA yield as high as whole blood sampling, they are minimally invasive and typically acceptable to the public, in particular for use in needle phobic patients and in control participants where risks associated with phlebotomy may be unacceptable when not otherwise undergoing phlebotomy or treatment themselves. DNA was extracted from the ORAcollect buccal swabs using a process of ethanol precipitation.

Figure 18: DNA Genotek ORAcollect OCR-100 buccal swabs

Extraction of DNA from ORAcollect buccal swabs involved initial incubation of samples at 50 °C for a minimum of two hours. This step ensured adequate release of DNA and inactivation of residual nucleases. Following incubation, each 1000 µL sample was divided in two, with one half destined for inclusion in the working stock of DNA and the remaining half frozen at -80°C in the Discipline of Surgery biobank stock. 20 µL of PT-L2P, 'prepIT' solution was added to each 500 µL aliquot and mixed. This resulted in a turbid sample, due to the desired precipitation of impurities. Following incubation in a 4°C fridge for 10 minutes, each sample was centrifuged at room temperature for 8

minutes at 15,000 g. The resulting clear supernatant was transferred to a new microcentrifuge tube with the remaining pellet containing impurities was discarded. 600 µL of room temperature 95-100% ethanol was then added to the clear supernatant and mixed gently by inversion 10 times to precipitate the DNA and allow homogenous distribution throughout the sample. Following a 10 minute period at room temperature, the sample was again centrifuged at room temperature for two minutes at 15,000 g, leaving the concentrated DNA in a pellet at the base of the tube. The clear supernatant which then contained impurities was discarded, leaving the manually purified DNA pellet behind. Next, 250 µL of 70% ethanol was used to wash the DNA pellet and this was poured off and allowed to air dry to ensure all ethanol was removed. Finally, 100 µL of TE solution, a DNA storage buffer containing 10 mM Tris-HCl and 1 mM EDTA and with a pH of 8.0, was added and then vortexed for 10 seconds, prior to DNA storage. To ensure complete rehydration of the DNA, the sample was then air incubated at 50°C for 1 hour with occasional mixing, which completed the DNA extraction process. The samples were then stored at 4°C for working stock and in -20°C freezers for longer-term storage. Samples for inclusion in the biobank, not for early use, were stored in -80°C freezers.[171]

2.2.2 EDTA-stabilised whole blood

Samples of EDTA-stablised whole blood were extracted in an automated fashion using the Roche MagNA Pure Compact (MPC) robotic instrument. The MPC utilises a magnetic bead technology for the isolation process of DNA (Figure 19), resulting in a typical DNA yield of 15 – 28 µg per 1,000 µL sample of mammalian whole blood.[172] Magnetic beads are a simple and reliable method of purifying genomic DNA whereby DNA selectively binds to the surface of magnetic beads, while other contaminants stay in solution. The MPC system uses protein denaturant to lyse and inactive nucleases within the sample and silica-coated magnetic beads, which exhibit effective nucleic acid-binding properties.[173] The automated MPC process included 1) sample preparation with nuclease inactivation, 2) separation of DNA from the target solution (by binding to the silica-coated magnetic beads), 3) washing, and 4) DNA elution into a buffer solution.[174-176] Ready-to-use nucleic acid isolation kits with prefilled cartridges allowed isolation of nucleic acids from up to eight samples per cycle.

The procedure for each extraction began with placement of the Nucleic Acid Isolation Kit I, which is the appropriate set used for DNA extraction from mammalian whole blood samples of up to 1mL per sample. The isolation kit's barcode was scanned to identify the kit used. The cartridge rack and tube rack (with elution tube rack) were removed from the MPC instrument, and the sample tubes placed

into the appropriate row, each containing 1mL of whole blood for extraction. The elution tubes were also placed, and each barcode scanned to identify its place within the rack. Unique sample identifiers (Shire reference numbers) were added for each sample. An appropriate number of tip trays (one per purification) were also assigned into the appropriate positions. Following selection of the appropriate purification protocol, DNA_BLOOD-1000, the next steps involved proceeding to the confirmation screen, closing the MPC lid and beginning the extraction process. On completion of extraction, the elution tubes containing extracted and purified DNA were removed for storage, and the sample tubes, tips and isolation kit discarded. The samples were then stored at 4°C for working stock and in -20°C freezers for longer-term storage. Samples for inclusion in the biobank, not for early use, were stored in -80°C freezers.

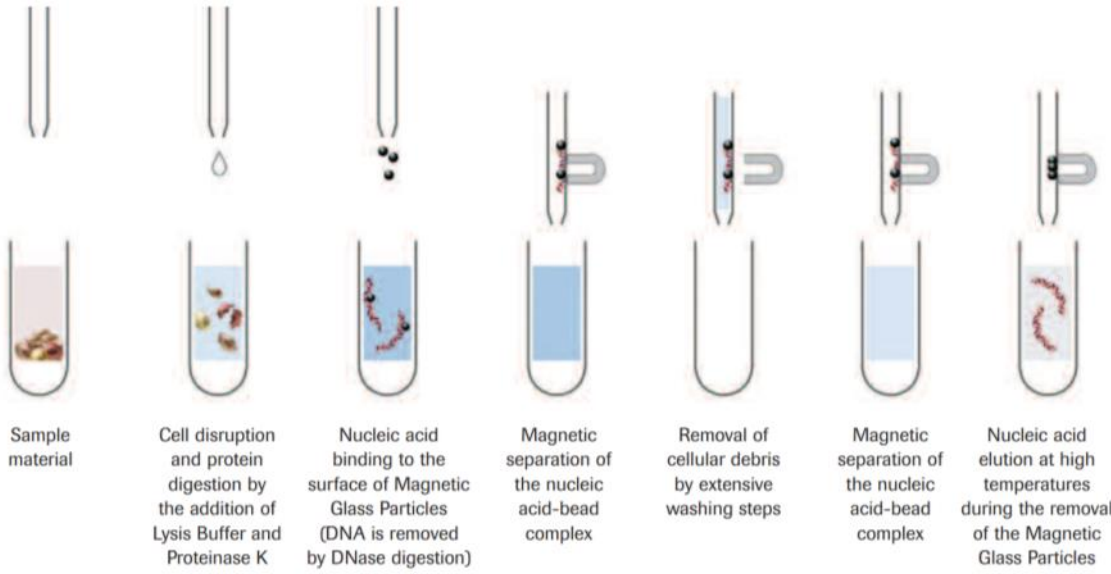


Figure 19: Schematic of nucleic acid purification using the Roche MagNA Pure Compact instrument (Image reproduced from Roche)[172]

2.3 Quantification of DNA concentration and purity

Following DNA extraction from Genotek ORAcollect-buccal DNA swabs or from EDTA-stabilised whole blood, evaluation of the DNA concentration and purity was undertaken. This was performed using absorbance spectroscopy, which is a common method of DNA quantification that uses a photo-detector to measure the amount of light that passes through a given specimen at a particular wavelength of interest. Variations in the intensity of light transmitted through a sample give an indication of the concentration of the absorbing molecule, as per the Beer-Lambert Law.[177] Nucleic acids have absorbance maxima at 260 nm; therefore the intensity of 260 nm light transmitted through a sample was measured, to calculate the concentration of DNA within each sample. Results

were compared with the extent of absorbance through a blank reference sample. The following Beer-Lambert Law equation correlates calculated absorbance with concentration:[178]

$$DNA\ Concentration\ (ng/\mu L) = A_{260} \times E \times b^{-1}$$

A_{260} is the absorbance at 260 nm wavelength represented in absorbance units (A), E is the wavelength-dependent molar absorptivity coefficient (or extinction coefficient) with units of litre/mol.cm (0.020 for double-stranded DNA), b is the instrument-specific path length in cm.

Following assessment of DNA concentration, it was also desirable to estimate the quality of DNA and the extent of impurities in the samples. Nucleic acid samples are commonly contaminated with other solutes such as proteins or organic compounds from accidental contamination or residual chemical residues from the DNA extraction process. The technique of absorbance spectroscopy provided the added benefit of being able to measure sample purity as well as nucleic acid concentration. Historically, the quality of nucleic acid samples was assessed by calculating the ratio of sample absorbance at 260 nm : 280 nm ($A_{260/280}$), since proteins, in particular aromatic amino acids, tend to absorb light at 280 nm. Samples with $A_{260/280}$ measuring ≈1.8 are typically accepted as 'pure' for DNA samples, while an $A_{260/280}$ ≈2.0 are regarded as a pure for RNA samples.

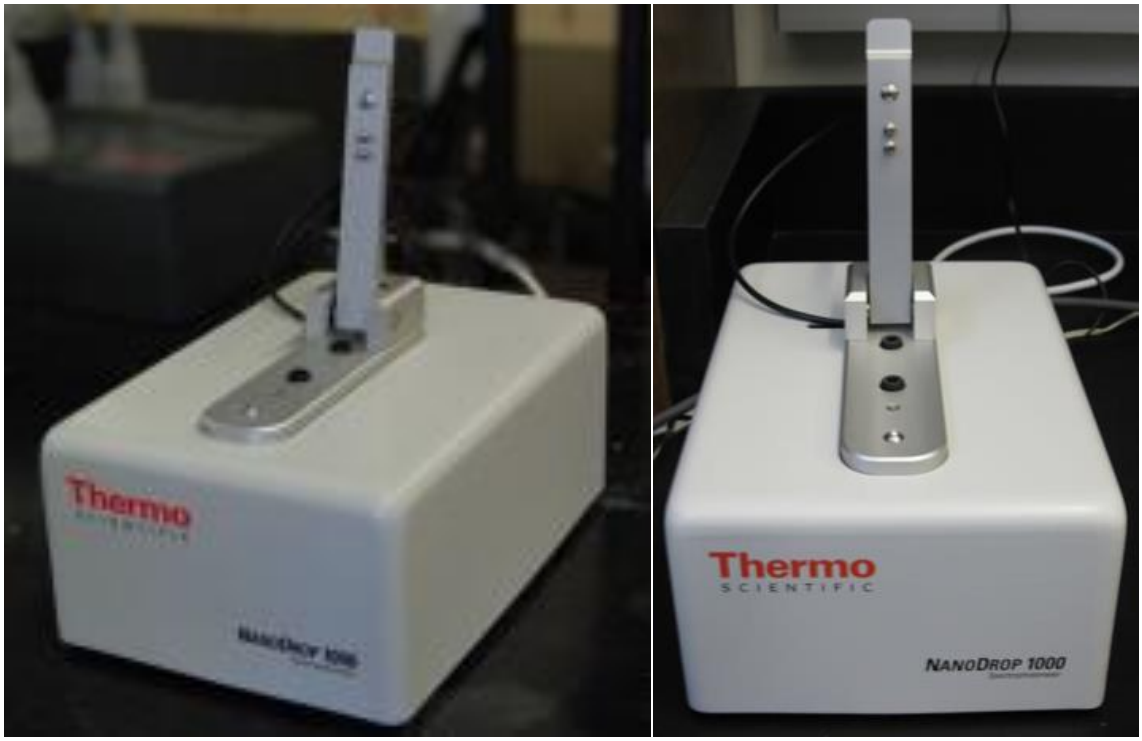


Figure 20: Thermo Scientific NanoDrop™ 1000 Spectrophotometer

The process for measurement of DNA concentration and purity using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Figure 20) was undertaken as follows:[178,179]

The NanoDrop software was opened and the 'Nuclei Acid' Protocol selected. Thermo Scientific recommend the 'DNA-50' setting which specifies a proprietary constant used for double-stranded DNA. The sampling arm was opened and the fibre optic surface and the lower pedestal cleaned with deionised water. 1 µL of pure prepIT•L2P DNA buffer solution was pipetted onto the lower fibre optic pedestal. The sampling arm was closed, bringing the photodetector for spectral measurement into contact with the fibre optic surface with the sample bridging the gap between the two. A 'blank' measurement was then performed using this 1 µL sample of the DNA buffer solution, within which all DNA samples were suspended. When the measurement was complete, the concentration reading was zeroed 0.0 ng/µL.

Following this initial calibration, the blanking buffer was cleaned from both pedestals and the Spectrophotometer was prepared for sample assessment. For each sample, 1 µL was pipetted onto the lower measurement pedestal, the sampling arm closed and the spectral measurement performed. DNA concentration, in ng/µL along with the $A_{260/280}$ was then recorded for each specimen and a graph of 10 mm absorbance Vs. wavelength was generated. These graphs illustrate the absorbance profile of each sample and facilitate manual quality control; a pure DS DNA sample generates a peak at 260 nm (Figure 21).

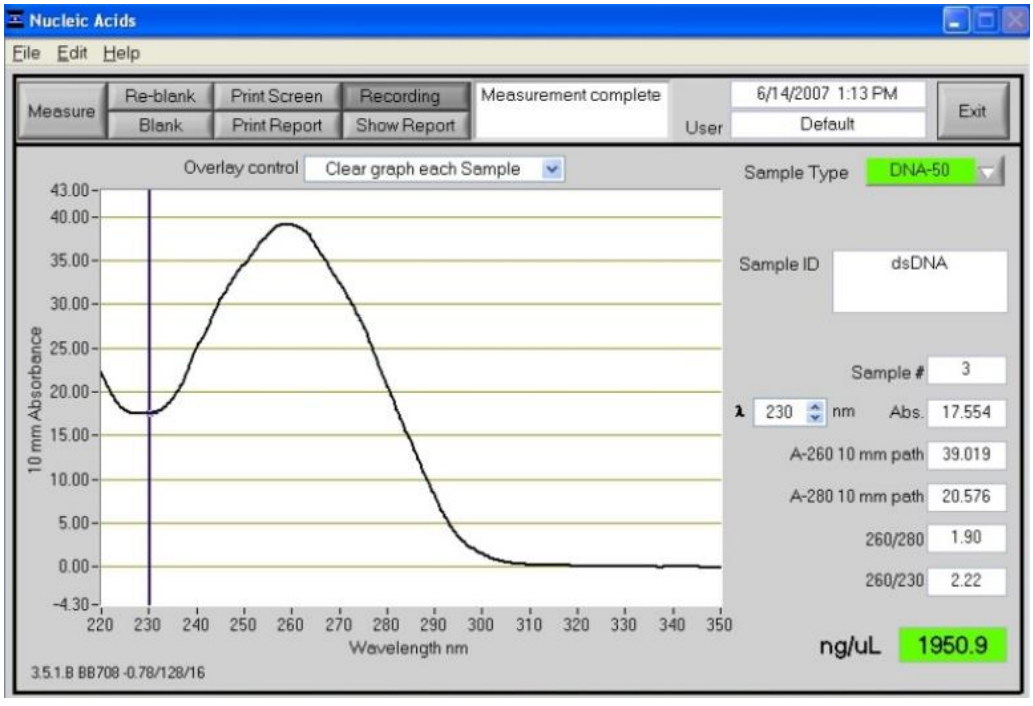


Figure 21: Sample screen output from Thermo Scientific NanoDrop™ 1000 Spectrophotometer. Note DNA concentration of 1,950.9 ng/µL, $A_{260/280}$ = 1.9 and graph peak at 260 nm for this sample.

2.4 Polymerase chain reaction and genotyping

The assessment of extracted DNA for the presence of single nucleotide polymorphisms required initial sample dilution to a standardised DNA concentration, subsequent real-time polymerase chain reaction (RT-PCR) to amplify the targeted DNA using sequence-specific primers, and assessment of polymorphism-specific genotype by allelic discrimination using the Applied Biosystems TaqMan platform. All DNA genotyping experiments were undertaken using either the Applied Biosystems StepOnePlus Real-Time PCR System or the 7900HT Fast Real-Time PCR System, also from Applied Biosystems.

The TaqMan platform draws from a collection of over 4.5 million assays to detect specific human genome SNPs.[180] The TaqMan method of SNP analysis is most suitable for the candidate gene approach, where a small number of SNPs are examined in a large cohort, as is required for the work described in this thesis; it is time-efficient and cost-effective with a high throughput, accuracy, and precision.[181] Each SNP-specific assay used in this work contained a VIC dye-labelled probe, a FAM dye-labelled probe, and two target-specific primers. The wild-type SNP allele and the mutant SNP allele were each targeted using these region specific forward and reverse primers. The TaqMan probes used fluorescent reporter dyes, VIC and FAM, which attach to the 5' end, with the complementary quencher dye attaching to the 3' end. Amplification was performed using a thermal cycling RT-PCR system. Throughout amplification, each probe bound to its associated allele of interest and the TaqMan polymerase enzyme cleaved the bound probe, generating a fluorescent signal which was interpreted to determine the genotype.[180] Allelic discrimination plots were then generated, with fluorescence of FAM and VIC plotted on X and Y axes to visually demonstrate the three genotypes. Manual quality control of the automatic genotype calling was performed using multicomponent plots.[180,182]

The stepwise process for genotyping using the 7900HT Fast Real-Time PCR System involved initial pre-dilution of DNA samples with nuclease-free water (NFW), such that the concentration of each sample measured 4.44 ng/µL, resulting in 40ng of DNA per 9 µL, which was convenient for plate set-up. All reagents and a 96 well TaqMan Genotyping Plate mounted on an ice block, were prepared inside a PCR extraction cabinet. Required diluted DNA samples, the TaqMan Universal Master Mix and the specific TaqMan SNP Genotyping Assay were removed from the fridge/freezer and allowed to thaw.

1000 µL of Master Mix was initially added into the 100 µL TaqMan SNP Assay such that all reagents could be conveniently pipetted into each plate well in the following proportions:

• Master Mix + TaqMan SNP Assay	11 µL	Wells 1 – 96
• DNA samples pre-diluted with NFW	9 µL (40ng DNA)	Wells 1 – 92
• NFW control sample	9 µL	Well 93
• Homozygous wild-type control	9 µL	Well 94
• Heterozygous control	9 µL	Well 95
• Homozygous variant control	9 µL	Well 96

Non-template control samples were used and were consistently located in wells 93 – 96. These controls ensured quality control by orientating the VIC and FAM dye clusters to an origin and acted as controls for detection of any DNA contamination.

Following plate preparation and sealing, PCR Amplification was initiated using the ABI 7900 HT Fast Real-Time PCR System. The plate was loaded into the machine and a new project with a 96-well plate and the assay >ΔΔCt(RQ) was selected using the Applied Biosystems software. A standard setup was selected, with three stages and 40 thermal cycles, which could be increased if amplification was noted to occur at a late stage on subsequent multicomponent plots. The required detectors were matched to the appropriate nucleotide SNP alleles, C, A, T or G and the case vs control wells were identified prior to commencing the run.

Subsequent to PCR Amplification, allelic discrimination was then performed to ascertain the genotype of each sample with respect to the relevant SNP being examined. The 96-well plate ewas again loaded into the ABI 7900 HT Fast Real Time PCR System and the marker relevant to the SNP being studied was selected and copied into the plate document. All required wells were identified and post-read analysis performed. The software then automatically identified the genotype of each sample on the plate, including the controls, based on the fluorescence identified and subsequently generates an allelic discrimination plot to illustrate the genotypes, with all samples overlaid on the same graph (Figure 22).

The automatic result generated for each point was then manually checked by reviewing the multicomponent plot for each sample (Figure 23), which depicts the spectral distribution of each dye in the selected wells over the duration of the PCR run. The relevant FAM and/or VIC plots correspond to the highest fluorescence depending on the genotype. Multicomponent plots were also quality checked for the known controls to ensure accuracy. Finally, all genotype results were exported to a Microsoft Excel file for analysis.

PCR amplification and allelic discrimination is a two-step process using the 7900HT Fast Real-Time PCR System. The process is broadly similar although is undertaken in one automated step using the Applied Biosystems StepOnePlus Real-Time PCR System or the 7900HT Fast Real-Time PCR System.[183]

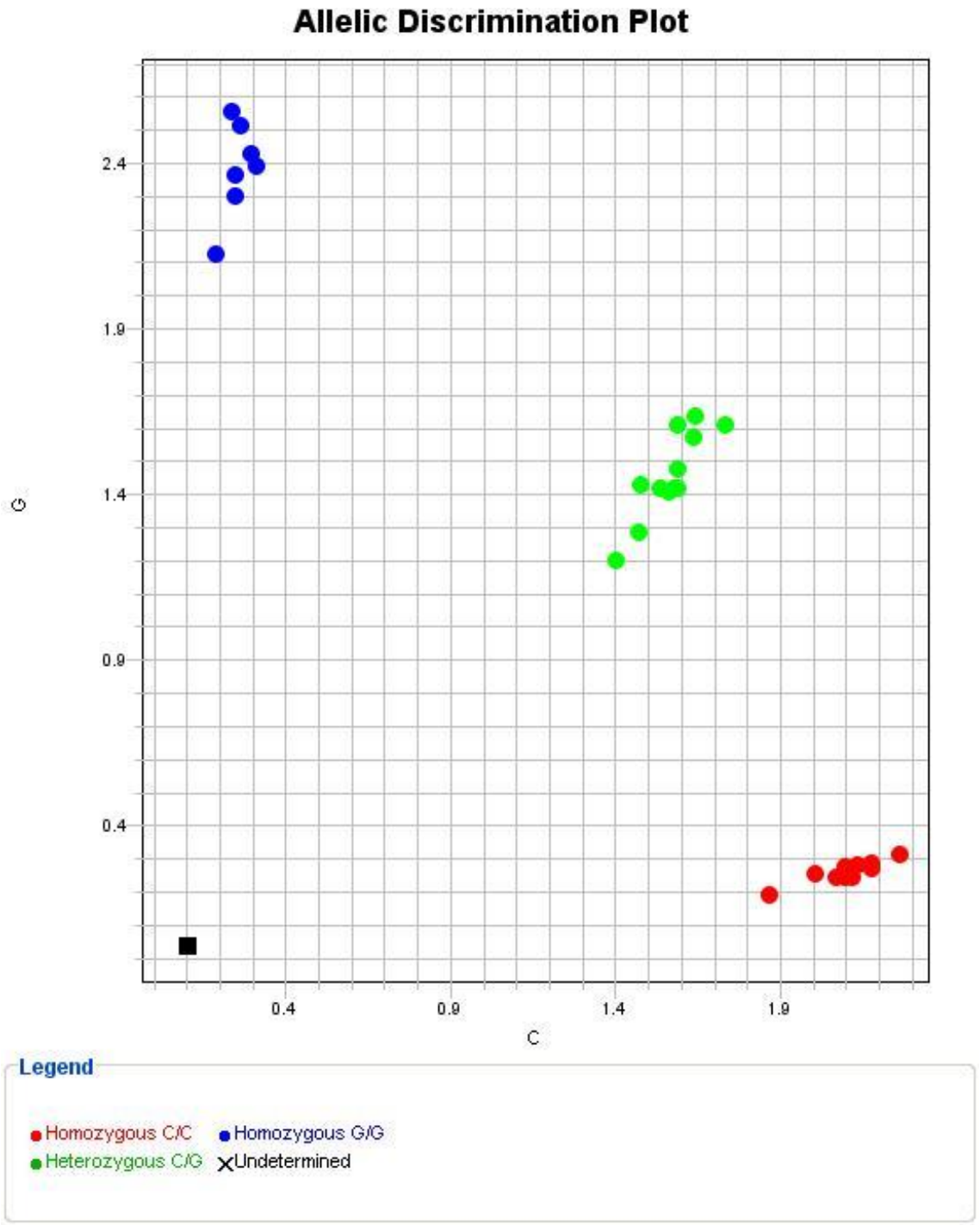


Figure 22: A Typical three-cluster allelic discrimination plot for a G → C SNP. Note the clusters of wild –type genotypes (blue), heterozygotes (green) and rare homozygous carriers (red). Also note the undetermined data-point near the origin representing the NFW control sample (Well 93).

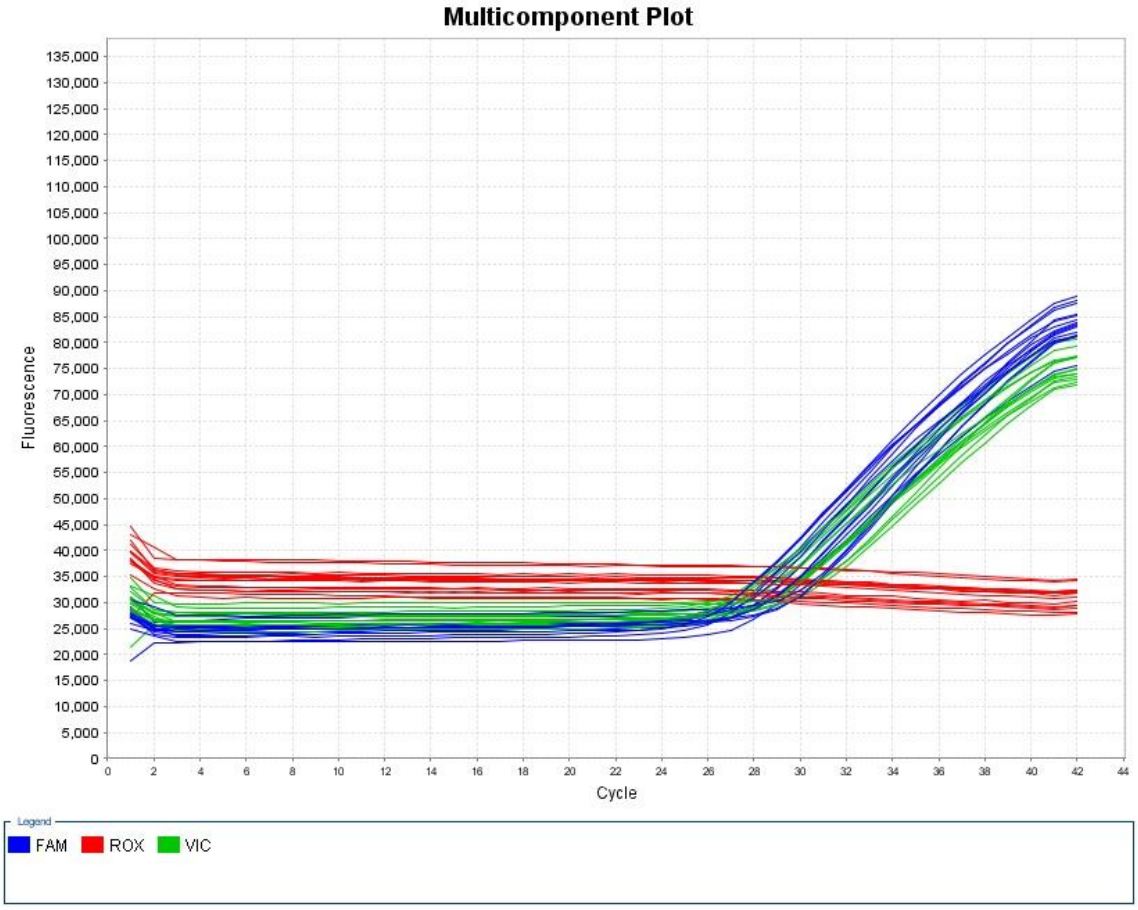


Figure 23: Example Multicomponent Plot demonstrating the spectral contribution of each sample's dye for a given well during the PCR run. Note that ROX dye is used as a passive internal reference.

2.5 Statistical analysis

Data recording and statistical analysis were performed using Microsoft Excel 2010 and IBM SPSS v22. Parametric tests and means ± standard deviation were utilised for normally distributed data while non-parametric tests and medians with range were utilised for non-normally distributed data. Pearson's chi-squared tests were used to compare distributions of categorical variables, while one-way ANOVA with Tukey post-hoc analysis was utilised for comparisons involving three or more categorical, independent samples. Hardy-Weinberg Equilibrium testing of biallelic SNPs was performed using the Pearson's chi-squared test. Chi-squared tests were also used for case-control genetic association analysis.

3 Chapter 3 - Clinical patterns of thyroid cancer presentation and review of management strategies in an Irish population.

3.1 Introduction

Thyroid cancer is the most common endocrine malignancy, accounting for 1% of all cancers, with an incidence of 162 cases per year in Ireland.[3] Most recent published data from the National Cancer Registry Ireland (NCRI) demonstrate an increasing incidence of thyroid cancer from 60 per year during the mid 1990's to more than 160 cases per year during the late 2000's.[3] Similar trends have also been demonstrated in other populations, including the United Kingdom, the United States and South Korea.[23,90,184] Owing to this rapid increase in incidence, thyroid cancer is projected to replace colorectal cancer as the fourth leading cancer diagnosis in North America by 2030.[7] This observed increased incidence primarily comprises the more indolent differentiated thyroid cancers (DTC), typically in early disease stages, and crucially has not been accompanied by a concomitant increase in mortality, suggesting potential over-diagnosis of an entity that, if undetected, would otherwise not have resulted in symptoms during a person's life or affected their mortality.[89-92] DTC has an excellent prognosis in the majority of cases; data from the US Surveillance, Epidemiology, and End Results (SEER) population-based database examining 51,061 DTC patients treated between 2004 and 2012, demonstrates overall survival and cancer-specific survival rates of 96.5% and 98.8% respectively at 5 years.[185] In Ireland, the 5 year survival rate for all thyroid cancer has improved from 71% (1994-1998) to 91.8% (2010-2014), while survival was best for differentiated subtypes; patients diagnosed with DTC between 2005 and 2009 demonstrated 5 year survival rates of greater than 98%.[3] The mainstay of thyroid cancer treatment involves surgical resection with or without adjuvant RRA; it follows that over-diagnosis of DTC may give rise to potentially avoidable physical and psychological morbidity secondary to interventions, including hypoparathyroidism and hypocalcaemia, recurrent laryngeal nerve injuries and dysphagia. Increased rates of thyroid nodule detection, rather than a true increase in disease incidence, may be attributable to wider access to healthcare services in high income countries in addition to improving diagnostic accuracy of ultrasonography, computed tomography, and magnetic resonance imaging.[92]

Key recommendations from British Thyroid Association (BTA) Guidelines for the Management of Thyroid Cancer 2014 include diagnostic lobectomy for those with Thy 3 or Thy 4 fine-needle aspiration cytology (FNAC). Total thyroidectomy is advised for patients with Thy 5 FNAC or with confirmed DTC following diagnostic lobectomy where tumour size exceeds 4 cm, or measures any

size in association with specific risk factors including multifocal disease, bilateral disease, extra-thyroidal spread (pT3 and pT4a), familial disease, and those with clinically or radiologically involved nodes and / or distant metastases. Lobectomy alone is deemed sufficient for patients with unifocal papillary microcarcinoma (microPTC, <1 cm) without risk factors; these include multifocality, larger size (6-10mm), extra-thyroidal extension, poor differentiation and a desmoplastic fibrosis or an infiltrative growth pattern. There is a paucity of well designed, peer-reviewed randomised or prospective studies to support an advantage of total thyroidectomy over lobectomy in patients with unifocal tumours 1–4 cm in diameter, age <45 years, and without extra-thyroidal spread, familial disease, evidence of lymph node involvement, angioinvasion or distant metastases. In these cases, BTA guidelines recommend a "Personalised Decision Making" approach which advocates a shared doctor-patient decision making process in conjunction with multi-disciplinary team (MDT) input, with due consideration for recurrence risk, patient comorbidities, and personal circumstances and values.[52]

BTA Recommendations for the use of radioiodine remnant ablation (RRA) adopt a similar approach with RRA indicated for all patients with DTC >4 cm, or for those with tumours of any size in the presence of gross extra-thyroidal extension or distant metastases. Patients with tumours ≤1 cm without specific risk factors do not benefit from RRA. A "Personalised Decision Making" approach is again advised for those patients with tumours measuring 1 – 4 cm without risk factors.[52]

In this study we aim to describe the patterns of DTC presentation, treatment strategies and assess degree of adherence to current clinical practice guidelines (BTA 2014) in a cohort of patients treated for differentiated thyroid cancer at our institution. We further examine those patients where a "personalised decision making" approach is recommended and how this subset of DTC are managed in the context of equivocal guidelines.

3.2 Methods and materials

Data was prospectively recorded from consecutive patients attending a specialist thyroid cancer clinic in a tertiary referral centre (UHG) over a three year period between August 2014 and August 2017. Data collection was undertaken at the time of blood and sputum sample collection for inclusion in the UHG thyroid cancer BioBank. All patients provided informed written consent for inclusion, which was ethically approved by the UHG research ethics committee. Patient demographics and pathology parameters including tumour characteristics (histology / TNM staging

details) were recorded, in addition to management strategies with a focus on surgical decision making and the use of RRA. All patients included in the study were discussed at an endocrine surgery multi-disciplinary meeting typically attended by at least one endocrinologist, endocrine surgeon, radiologist, radiation oncologist and pathologist. Thyroid cancer staging parameters were designated as per the AJCC Cancer Staging Manual 7th Edition guidelines while FNAC results were reported using the UK Royal College of Pathologists (RCPath) terminology for thyroid cytology reporting.[100,186] The British Thyroid Association's 2014 'Guidelines for the Management of Thyroid Cancer' were accepted as best practice guidelines at the time of the analysis.[52] Data recording and statistical analysis were performed using Microsoft Excel and IBM SPSS v22. Chi square tests were used to compare means of categorical variables, while one-way ANOVA with Tukey post hoc analysis was utilised for comparing means involving three or more categorical, independent samples.

3.3 Results

Data was collected for 178 consecutive patients attending a dedicated thyroid cancer clinic over a three year period, between August 2014 and August 2017. 130 (73%) patients were female. The median age at diagnosis was 43.5 years old (range 15 - 83 years). 152 patients (85%) had papillary thyroid tumours while the remaining 26 (15%) patients had tumours with follicular histology. Median total tumour size was 26 mm (range 1 – 110 mm). Multifocal disease was present in 36% (n=64) of patients. Lymphovascular invasion and extracapsular extension were evident in 33% (n=58) and 31% (n=55) of tumours respectively. 48% (n=86) had at least one lymph node excised at surgery, with a mean harvest of 7.6 nodes (± SD 8.5). 34 patients were confirmed to have lymph node metastases on histological analysis, representing 40% of those with nodes excised and 19% of all patients. The average number of positive nodes retrieved was 4.7 (± SD 4.4).

FNAC results were available for 77% (n=137) of patients. The remaining 41 patients either did not have FNA performed or it was performed at another centre where results were inaccessible. A breakdown of FNAC results in relation to subsequent operative management is shown in Figure 24. All 13 patients with Thy 5 FNA results underwent primary total thyroidectomy. Primary thyroidectomy was also performed for 3, 6, 12 and 15 patients with Thy 1 – 4 FNA results respectively; of these 36 patients, 24 had features identified on pre-operative imaging that indicated total thyroidectomy, including size > 4cm, lymphadenopathy, extra-capsular extension or bilateral disease. Total thyroidectomy was performed for benign indications such as multinodular goitre or

Grave's Disease for nine patients, while three patients had no definitive indication recorded for upfront total thyroidectomy.

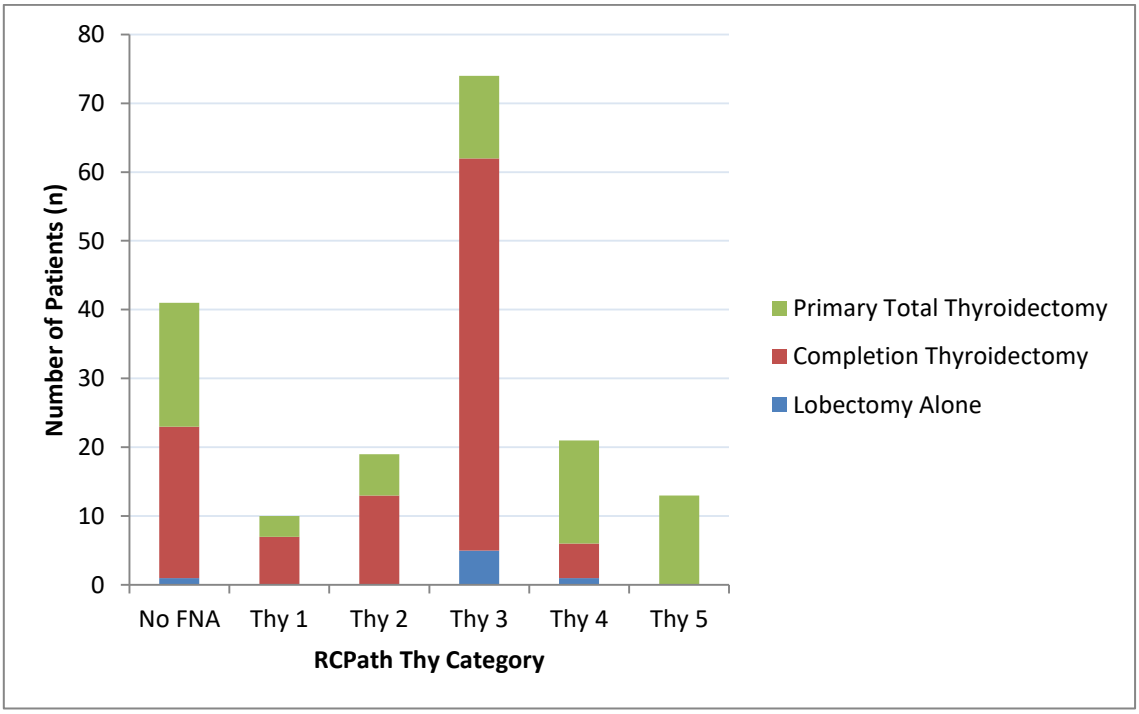


Figure 24: Surgical management per Royal College of Pathologists Thy category

3.3.1 Surgery

38% (n=67) of patients underwent primary total thyroidectomy while the remaining 62% (n=111) underwent thyroid lobectomy, with 95% of those (n=105/111) proceeding to completion thyroidectomy. Six patients (3%) had thyroid lobectomy alone (Table 4). Gender had an impact on choice of surgical management as determined by one-way ANOVA (p=0.009). Patients who had lobectomy alone were more likely to be male (68%, n= 4/6) compared to those who proceeded to completion thyroidectomy (27%, n=28/105) (p=0.031). Tumour size > 4cm, multifocality and extra capsular extension were all associated with an increased rate of completion thyroidectomy (p=0.044, p=0.027, p=0.044). Final histological subtype and patient age did not appear to influence choice of surgical management in this cohort (p=0.246, p=0.793).

	Primary Total Thyroidectomy	Lobectomy Alone	Lobectomy Followed by Completion Thyroidectomy
Number of patients	67 (38%)	6 (3%)	105 (60%)
Female	51 (76%)	2 (33%)	77 (73%)
Age, years	46 ± 16	50 ± 23	46 ± 16
Preoperative FNAC Thy 1	3 (5%)	0 (0%)	7 (7%)
Preoperative FNAC Thy 2	6 (9%)	0 (0%)	13 (12%)
Preoperative FNAC Thy 3	12 (18%)	5 (83%)	57 (54%)
Preoperative FNAC Thy 4	15 (22%)	1 (17%)	5 (5%)
Preoperative FNAC Thy 5	13 (19%)	0 (0%)	0 (0%)
No Pre-operative FNAC	18 (27%)	0 (0%)	23 (22%)
Papillary (final histology)	61 (91%)	5 (83%)	86 (82%)
Follicular (final histology)	6 (9%)	1 (17%)	19 (18%)
Size, mm	22 (2-110)	5.25 (2-65)	30 (1-110)
Multifocal	26 (39%)	0 (0%)	38 (36%)
Nodal disease	25 (37%)	0 (0%)	9 (9%)
Lymphovascular invasion	29 (43%)	1 (17%)	28 (27%)
Extracapsular extension	23 (34%)	1 (17%)	31 (30%)

Values presented as n (%), mean ± SD, or median (range). FNAC, fine-needle aspiration cytology; TT, total thyroidectomy.

Table 4: Clinico-pathological parameters grouped by surgical procedure

94% of patients (n=168/178) were surgically managed in strict adherence with recent BTA guidelines (Table 5). Of those patients who had surgical management not strictly aligned with guidelines (n=10), one had a tumour > 4cm at lobectomy, but declined completion thyroidectomy and was lost to follow-up. The remaining nine patients had tumours < 1cm without risk factors, of which four had primary total thyroidectomy due to concurrent multi-nodular goitre causing dysphagia, respiratory

compromise or unacceptable aesthetic appearance. Five patients proceeded to interval completion thyroidectomy following lobectomy where not indicated by guidelines; two patients had completion thyroidectomy due to personal preference influenced by their family history of thyroid tumours (one benign and the other malignant); another patient had interval radiological lymphadenopathy and recent history of metachronous laryngeal cancer; while the remaining two patients proceeded to completion thyroidectomy based on patient preference, without family history and each with age>45 being their only relative risk factor for recurrence. The rate of surgical management in agreement with BTA guidelines was not significantly affected by gender (p=0.824), age >45 years (p=0.158) or histological subtype (p=0.671). However, tumour size did have a significant impact; patients with tumours measuring <1cm were most likely to undergo surgical management differing from 2014 BTA recommendations (p<0.001).

	Patients (%)	Compliant with BTA Guidelines for Surgery (%)
All Patients	178 (100%)	168 (94%)
≤1cm	27 (15%)	18 (67%)
1–4cm	113 (63%)	113 (100%)
>4cm	38 (21%)	37 (97%)
Multifocal	64 (36%)	64 (100%)
Lymphovascular invasion	58 (33%)	57 (98%)
Extra-capsular extension	55 (31%)	54 (98%)
Age >45	86 (48%)	79 (92%)

Table 5: Surgical management compliance with BTA 2014 guidelines

Following thyroid lobectomy, "personalised decision making" was the recommended strategy for the surgical management of 32 patients based on BTA guidelines (Table 6). 24 patients had intermediate size tumours (1 – 4 cm) without risk factors, four patients had microPTC measuring 6 - 10 mm, while four patients with microPTC exhibited multifocal disease. A more aggressive, treatment-driven approach was typically favoured by patients and the multidisciplinary team with 97% (n=31/32) of patients proceeding to completion thyroidectomy; one patient with a unifocal 15mm papillary thyroid tumour without risk factors had lobectomy alone.

3.3.2 Radioiodine remnant ablation

82% (n=146/178) of patients underwent adjuvant radioiodine remnant ablation. RRA was more likely to be administered in patients with tumour size >4cm (p<0.006), node positivity (p=0.002), LVI (p<0.001) or extracapsular extension (p=0.001). No statistically significant impact on RRA utilisation was demonstrated by gender (p=0.547), age >45 years (p=0.203), histological subtype type (p=0.139) or tumour multifocality (p=0.067).

In relation to BTA guidelines, 43% (n=77) of all patients had a definitive indication for RRA, while 12% (n=21) had a definitive recommendation against RRA. A "Personalised decision making" approach was recommended for 45% (n=80) of patients, with 66 out of those 80 patients (83%), progressing to treatment with RRA (Table 6). Overall, 97% (n=172/178) of RRA treatment decisions, were in accordance with 2014 BTA guidelines; one patient was recommended for RRA due to extra-capsular extension but declined, while the remaining six patients received RRA outside of current BTA recommendations. None of these six patients had definitive indications for RRA based on 2014 BTA guidance, however two patients had weaker risk factors for recurrence; one had multi-focal disease in one lobe although measuring ≤1cm in total size, while another was >45 years old at diagnosis. The rate of agreement with 2014 BTA RRA recommendations was not significantly affected by gender (p=0.721), age >45 years (p=0.115) or histological subtype (p=0.884), whereas patients with tumours measuring <1cm were likely to undergo RRA management differing from BTA recommendations (p<0.001) when compared to patient in the 1-4 cm or >4 cm tumour size subgroups.

	"Personalised Decision Making" Recommended for Surgical Management (n=32)	"Personalised Decision Making" Recommended for RRA Management (n=80)
Had RRA / thyroidectomy	31 (97%)	66 (83%)
Female	25 (78%)	66 (83%)
Mean Age (± SD, yrs)	37 (± 10)	43 (± 15)
Papillary	28 (88%)	70 (88%)
Follicular	4 (13%)	10 (12%)
Median Size (range, mm)	21 (2 – 40)	22 (10 – 40)
≤1cm	8 (25%)	1 (1%)
>1 cm, ≤ 4 cm	24 (75%)	79 (99%)
>4 cm	0 (0%)	0 (0%)
Multifocal	4 (all microPTC)	30 (38%)

Table 6: Clinico-pathological details for patients where the BTA recommend a "Personalised Decision Making" approach

3.4 Discussion

This study assessed practice patterns in the management of DTC in an Irish tertiary referral centre for thyroid cancer, with a particular focus on patients with intermediate size, low risk tumours where a paucity of high-level evidence prevents current guidelines from supporting a definitive therapeutic approach. Overall, DTC treatment strategies were largely in agreement with best practice recommendations. Concordance with 2014 BTA guidelines was demonstrated in 94% of surgical, and 97% of RRA therapeutic decisions. In the minority where discordance with guidelines was demonstrated, there was a tendency towards over-treatment, with 9 out of 10 surgical treatment decisions, and 5 out of 6 RRA treatment decisions, resulting in the more aggressive treatments of total thyroidectomy and radioiodine therapy where lobectomy and no RRA would have been recommended by the guidelines respectively. Early studies examining the appropriate surgical management of DTC initially suggested a one-size fits all approach with total thyroidectomy resulting in lower recurrence rates and improved survival for all patients with DTC > 1cm.[187,188] More recently, increasing recognition of patient and disease prognosticators has facilitated improved risk

stratification such that low risk patients, even with tumours > 1cm, may be treated with a more selective and individualised approach while maintaining improved outcomes and reducing treatment related morbidity.[84-87] This is borne out by incremental guideline amendments towards a more conservative approach in recent best practice recommendations. The 2009 American Thyroid Association (ATA) and 2006 European Thyroid Cancer Consensus guidelines both previously recommended total thyroidectomy for all DTC >1 cm, while more recent 2014 BTA and 2015 ATA guidelines now suggest that lobectomy alone is an option for those with tumours 1 - 4cm without risk factors. Emerging evidence from Japan and the USA has also recently established the safety of choosing active surveillance for papillary microcarcinomas, with interval growth of these tumours over a 5 year period observed in less than 15% of patients, at which point surgical intervention may be indicated.[189,190] Furthermore, improved identification and classification of more indolent histological subtypes of DTC may also improve risk stratification and reduce over-treatment of less aggressive phenotypes; the encapsulated follicular variant of papillary thyroid carcinoma (fvPTC) has recently been re-designated as a non-invasive follicular thyroid neoplasm with papillary-like nuclear features, effectively reclassifying this subgroup as non-cancerous, potentially requiring a less aggressive therapeutic approach to both surgery and RRA.[15]

Almost half of the patients in our cohort were subject to equivocal recommendations for either surgery or RRA, based on BTA guidelines, whereby a “personalised decision making” approach was suggested. These patients typically had intermediate-sized tumours without specified risk factors for recurrence. Despite large retrospective cohort studies, the rarity of mortality and disease recurrence events associated with this patient group poses a challenge for provision of definitive evidence-based guidelines. In the absence of specific recommendations, the “personalised decision making” approach encourages consideration of patient factors such as age, comorbidity, performance status, patient preference, ability to engage with follow-up for the contralateral lobe and the impact of increased surgical complication risk such as laryngeal nerve injuries and hypocalcaemia. In addition, tumour parameters tending towards guideline cut-offs may also be relevant, such as tumour sizes approaching 1 or 4 cm or tumour distance from the thyroid capsule. Clinician factors such as clinician preference and complication rates should also be considered. Almost all patients in our cohort who were recommended for a “personalised decision making” approach with regards to the need for completion thyroidectomy (n=32) and RRA (n=80), proceeded to the more aggressive options of completion surgery and RRA. These therapeutic decisions are likely an attempt to reduce potential DTC mortality and recurrence risk, however, the absence of sufficient evidence to support a definitive treatment course in these patients despite multiple large retrospective studies, suggests that any benefit gained by this strategy is likely to be small.[191] Furthermore, the relative

effectiveness of treatment strategies for recurrent DTC are such that the lower risk of recurrence for many patients in the "personalised decision making" cohort is acceptable given the benefit of avoiding a second surgery and exposure to radioiodine therapy. Potential benefits gained by the more aggressive options may be outweighed by both physical and psychological morbidity. The impact of extended patient waiting times, from initial diagnostic imaging and FNAC to thyroid lobectomy, subsequent completion surgery and onward to RRA, is extensive, and results in substantial psychological morbidity.[192] Physical complications from thyroid surgery are also substantial and include transient (8%) and permanent (2%) hyperparathyroidism, permanent (1%), transient (2%) and diplegic (0.4%) palsies of the laryngeal recurrent nerve (1%), superior thyroid nerve injury (4%) and dysphagia (1%). In particular, laryngeal recurrent nerve injury can have a significant social and psychological effect, while the management of hypoparathyroidism following completion thyroidectomy is often challenging, particularly for young persons and pregnant women.[193] Other than physical and psychological effects, completion surgery of potentially indolent disease also comes with financial implications; thyroid lobectomy is estimated to cost €5,277 per patient in Europe, including pre-surgical work-up, follow-up, and management of complications over 12 months.[194] This is in addition to the lifelong cost of thyroxine replacement, follow-up and monitoring associated with total thyroidectomy. In New South Wales, Australia, the increase in DTC treatment over 10 years up to 2012, for their 7.5 million population, has resulted in a reported AUD$ 18,600,000 in additional surgery-related health care expenditure.[195]

RRA is also associated with significant side effects. 20% of patients experience short term nausea, taste and smell impairment, and sialoadenitis. More significant complications include impairment of bone marrow and gonadal function and an increased risk of a second primary malignancy, for both solid and haematological cancers. 100 mCi (3.7 GBq) of radioiodine has been estimated to result in an extra 56 malignancies in 10,000 patients over 10 years, while Rubino et al observed a 30% dose-dependent increase in second primary malignancies following radioiodine treatment in a large multicentre cohort study of 6,841 patients treated for DTC.[194,196]

DTC is frequently quoted as having an excellent prognosis, however, there remains a significant risk of recurrence of between 5% and 30% and approximately 10% of patients die of this cancer.[185,197-199] This is reflected in the overall aggressive approach to the management of this cohort in cases of equivocal recommendations. Improved risk stratification measures are warranted to better identify these patients at risk of mortality and recurrence. Multiple risk factor assessment tools currently exist for estimating risk of mortality in patients with DTC and attempt to guide aggressiveness of management for those deemed low or high risk for mortality or recurrence. Commonly used DTC

prognostication systems include the AJCC/UICC TNM system (Tumour, Nodes Metastases), AMES (Age at presentation, Metastases, Extent, Size of primary), MACIS (Metastases, Age at presentation, Completeness of surgical resection, Extra-thyroidal Invasion, Size), EORTC (European Organisation for Research and Treatment of Cancer methodology), AGES (Age at presentation, Grade of tumour, Extent, Size of primary tumour).[200,201] These prognostication systems, using traditional demographic and staging parameters and focusing predominantly on mortality rather than recurrence risk, have largely failed to significantly guide management in a large subset of DTC patients as demonstrated by equivocal BTA 2014 guidelines for low risk intermediate size DTC.

Advances in molecular medicine have led to an improved understanding of the mechanisms of DTC carcinogenesis and risk indicators. Molecular markers including gene expression profiles, somatic gene alterations, and circulating biomarkers now provide improved indices for diagnosis, prognostication and therapeutic targets.[202] Increased understanding of the molecular pathogenesis of DTC has revealed alterations in the MAPK and PI3K–AKT major signalling pathways as primary pathogenic events in DTC carcinogenesis.[203] 2015 ATA guidelines now advocate consideration of $BRAF^{V600E}$ proto-oncogene mutation status, when it is known, in their modified risk stratification system, however testing is not routinely advocated outside of research settings. Guidelines also acknowledge the potential impact of various other mutations in genes such as BRAF, TERT, TP53, RAS, or PAX8/PPARγ although these are not formally included in risk assessment systems or recommended for routine testing.[58] Rather than testing for individual high impact mutations, a number of commercially available DTC genetic risk assessment tools analyse large panels of multiple mutations, each with smaller odds ratios for recurrence and mortality, but with promising overall accuracy. These include Afirma Gene Expression Classifier (mRNA expression of 167 different genes) and the Thyroseq Next-Generation Sequencing panel (DNA alterations in 14 genes, including >1000 mutations, and RNA alterations, including 42 fusions and 16 genes for expression). These molecular risk profiling adjuncts have demonstrated encouraging results in risk stratifying patients following nodule FNAC, in particular those with indeterminate cytology where lobectomy is then frequently performed as a diagnostic rather than therapeutic procedure.[204,205] In correctly identifying DTC from FNA cytology samples, ThyroSeq v2.1 has a reported sensitivity of 91% (95% CI 79 – 100), specificity of 92% (95% CI 86 - 98), and overall accuracy of 92% (95% CI 86 - 97).[205] This application of molecular risk stratification may help to reduce the number of diagnostic thyroid lobectomies undertaken; over one third of patients in our cohort had initial Thy 3 FNA results, with three quarters of these progressing to a two stage total thyroidectomy. However, while multiple studies have evaluated the utility of molecular testing for risk stratifying patients with indeterminate thyroid nodules and to

guide extent of initial thyroid surgery, there is a paucity of research examining the use of such markers in surgical and RRA management decisions post thyroid lobectomy in the case of DTC.[202,206]

3.5 Conclusion

DTC management in this cohort exhibited high levels of adherence to internationally recognised best practice guidelines. Where surgical and RRA therapeutic decisions did not satisfy BTA guidelines, a more aggressive management approach was usually adopted. A large proportion of patients are subject to a "personalised decision making" approach, owing to a lack of conclusive high level evidence to guide management. A tendency towards more aggressive surgical and RRA intervention was observed in this group. These findings highlight the requirement for improved risk stratification to rationalise management strategies to avoid overtreatment for patients who fall into indeterminate risk treatment groups.

4 Chapter 4 - Evaluation of the role of SNPs in hereditary susceptibility to differentiated thyroid cancer

4.1 Introduction

Thyroid cancer is the most common endocrine malignancy with an increasing incidence recorded over recent decades in Western countries, including Ireland. Genetic factors have been shown to play a significant role in the determination of thyroid cancer risk, more so than other cancers.[207] Epidemiological evidence arising from the Swedish Family-Cancer Database of 9.6 million people, estimates that 53% of thyroid cancer risk is accounted for by genetic factors, as opposed to environmental factors - the highest among all cancers studied.[114] Medullary thyroid cancer development is associated with point mutations in the RET proto-oncogene on chromosome 10, of which approximately 25% are germline mutations inherited in an autosomal dominant Mendelian pattern, as part of the Multiple Endocrine Neoplasia (MEN) 2A, MEN 2B, or Familial Medullary Thyroid Cancer (FMTC) syndromes.[208] While less is known about the sequence variations which confer an increased risk for sporadic DTC, it is unlikely that a similar high risk, high penetrance mutation will be found to underlie its pathogenesis. It is postulated that multiple mutations, each with relatively smaller effect sizes, together with environmental factors, are more likely to give rise to the majority of DTC.[209] Numerous somatic mutations have thus far been identified to play a role in DTC tumourigenesis. Better known examples include mutations in the mitogen-activated protein kinase (MAPK) pathway (Figure 11). The most common of these include BRAF V600E mutations in up to 60% of PTCs, RAS mutations in 15% of PTCs and RET/PTC, ALK or NTRK1 chromosomal rearrangements in 12% of papillary carcinomas.[108,110] Other implicated somatic mutations include TERT promoter, PIK3CA, AKT1 and TP53 mutations.[203]

In contrast to somatic mutations, germline hereditary factors which increase susceptibility to non-syndromic DTC are poorly understood. To date, 14 genes have been identified as increasing susceptibility to development of DTC not related to known hereditary syndromes; these include DICER1, FOXE1, PTCSC2, MYH9, SRGAP1, HABP2, BRCA1, CHEK2, ATM, RASAL1, SRRM2, XRCC1, TITF-1/NKX2.1 and PTCSC3. In addition, mutations at numerous loci which confer an increased DTC risk have been studied, although the associated causal genes remain unidentified. Many of these susceptibility loci and gene mutations confer inconsistent risk profiles when targeted genotyping methods are used to quantify their associated DTC risk in different populations, thereby necessitating further validation in multiple groups/populations.[210]

Single nucleotide polymorphisms arise due to nucleotide variation at a single position in a DNA sequence. Genome-wide association studies (GWASs) have identified numerous candidate SNPs which confer increased risk for the development of DTC.[211] Due to the geographical heterogeneity of DTC incidence and pathology, single-track candidate gene studies have subsequently been required to validate the candidate SNPs identified by GWAS in various other populations. One such SNP (rs965513, G>A) is located on 9q22.33, at the FOXE1 gene. The intronless FOXE1 gene encodes a protein called forkhead boxprotein E1, belonging to the forkhead family of transcription factors and plays a role in initiation of thyroid organogenesis including cell growth and growth-factor control of thyroid differentiation. Mutations in FOXE1 have been identified in patients with abnormal thyroid phenotypes including congenital hypothyroidism and thyroid dysgenesis. FOXE1 plays a role in thyroid tumour development, invasion and metastases, and has been identified as a gene of interest in thyroid cancer research with multiple thyroid cancer risk SNPs near FOXE1 recently elucidated.[212,213]

This study investigates the impact of a single nucleotide polymorphism, rs965513 at the FOXE1 locus, on susceptibility to differentiated thyroid cancer in a Western European patient population.

4.2 Methods and materials

A case control methodology was utilised to assess the impact of the candidate gene mutation (rs965513, G>A) on DTC risk. Allelic and genotypic frequencies among patients with confirmed DTC were compared with a control population, to assess the risk for DTC conferred by homozygous and heterozygous carriers compared to wild-type genotypes.

4.2.1 Patients & Samples

A thyroid cancer Biobank was first established at the Discipline of Surgery in the Lambe Institute for Translational Research as part of a multi-centre initiative, comprising clinico-pathological data and biological samples from patients attending endocrine cancer clinics at tertiary referral centres in the West of Ireland (University Hospital Galway) and in the South of France (Assistance Publique Hôpitaux de Marseille). Patients with histopathologically confirmed DTC over the age of 16 years were included. Patients with benign thyroid disease alone, medullary thyroid cancer or known familial non-medullary thyroid cancer were excluded. Patients with an a-priori diagnosis of any high-risk germline mutation were also excluded. Controls were recruited from the community and

comprised volunteers over the age of 60, without a personal or 1st degree family history of malignancy, excluding non-melanomatous skin cancer.

All patients and controls provided informed written consent before inclusion. Ethics approval for this study was granted following review by the local institutional Research Ethics Committee, at University Hospital Galway.

Demographic and clinico-pathological data was recorded at the time of specimen collection from patient self-reporting, electronic histopathology database and patient records. Two specimen collection methods were employed for this study; either a 10 mL sample of EDTA stabilised whole blood or via buccal swab salivary sample collection using a DNA Genotek Oragene 575 collection kit.

4.2.2 DNA Extraction & Genotyping

DNA was extracted from buccal swab and whole blood samples by manual ethanol precipitation. Following DNA extraction, evaluation of the DNA concentration and purity was performed with absorbance spectroscopy using the NanoDrop 1000 Spectrophotometer. Samples with a 260:280 nm (A260/280) absorbance ratio ≈ 1.8 were accepted as pure DNA.

DNA samples were initially diluted to a standardised DNA concentration, prior to real-time polymerase chain reaction (RT-PCR) to amplify the targeted DNA using sequence-specific primers, and assessment of polymorphism-specific genotype by allelic discrimination using the Applied Biosystems TaqMan platform. The TaqMan SNP-specific assay contains a VIC dye-labelled probe, a FAM dye-labelled probe, and two target-specific primers. The wild-type SNP allele and the mutant SNP allele are each targeted using these region specific forward and reverse primers. The TaqMan probes use fluorescent reporter dyes, VIC and FAM, which attach to the 5' end, with the complementary quencher dye attaching to the 3' end. Amplification is performed using a thermal cycling RT-PCR system. Throughout amplification, each probe binds to its associated allele of interest and the TaqMan polymerase enzyme cleaves the bound probe, generating a fluorescent signal which is interpreted to determine the genotype.[180] Allelic discrimination plots are then generated, with fluorescence of FAM and VIC plotted on X and Y axes to visually demonstrate the three genotypes. Manual quality control of the automatic genotype calling is performed using multicomponent plots. All DNA genotyping experiments were undertaken using either the Applied Biosystems StepOnePlus Real-Time PCR System or the 7900HT Fast Real-Time PCR System.

4.2.3 Statistical Analysis

Data recording and statistical analysis were performed using Microsoft Excel 2010 and IBM SPSS v22. Parametric tests and means ± standard deviation were utilised for normally distributed data while non-parametric tests and medians with range were utilised for non-normally distributed data. Pearson's chi-squared tests were used to compare distributions of categorical variables, while one way ANOVA with Tukey post-hoc analysis was utilised for comparisons involving three or more categorical, independent samples. Hardy-Weinberg Equilibrium testing of biallelic SNPs was performed using Pearson's chi-squared test. Chi-squared tests were also used for case-control genetic association analysis.

4.3 Results

277 patients with confirmed DTC and 309 non-cancer controls were genotyped. Patient and control groups were observed to be in Hardy-Weinberg equilibrium (X^2, p=0.09, p=0.07 respectively) (Figure 25). Patient demographics and clinico-pathological characteristics are detailed in Tables 7 & 8.

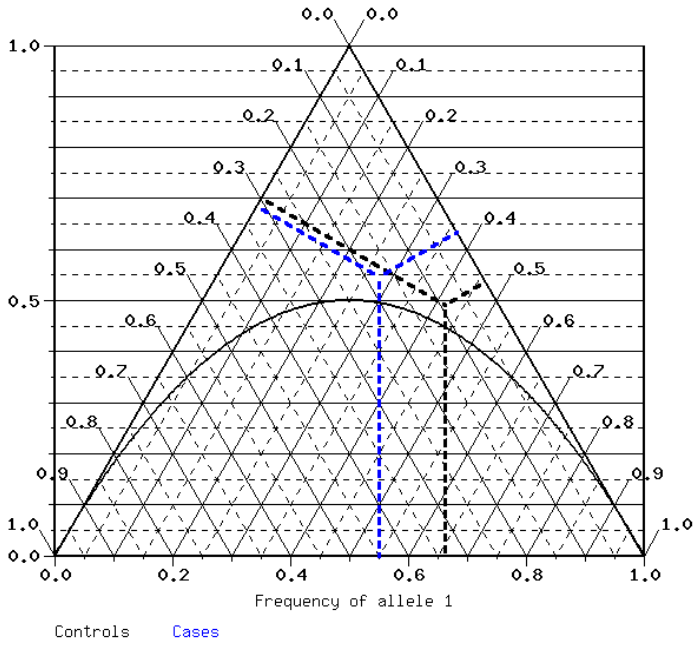


Figure 25: De Finetti ternary plot illustrating cases and controls in relation to the Hardy Weinberg Parabola for the FOXE1 Variant.[214]

Cases are represented by the blue dashed line, controls by the black dashed line. Length of vertical line: frequency of genotype GA; length of left perpendicular line: frequency of genotype GG; length of right perpendicular line: frequency of genotype AA; Intersection of Hardy-Weinberg parabola and vertical line: frequency of genotype GA in case of perfect Hardy-Weinberg equilibrium.

	DTC Cases n (%)	Non-cancer Controls n (%)
Patients	277 (100%)	309 (100%)
Gender		
Female	208 (75%)	252 (82%)
Male	69 (25%)	57 (18%)
European Caucasian:	255 (92%)	309 (100%)
Other (Asian, African):	22 (8%)	0 (0%)
Collection Site - France	109 (39%)	0 (0%)
Collection Site - Ireland	168 (61%)	309 (100%)
Age, at diagnosis / sampling		
Mean (+/- SD)	46.9 (+/- 14.95)	73.4 (+/- 8.68)
Median (range)	45 (16-84)	74 (23-100)

Table 7: Patient and control characteristics

	DTC Cases n (%)
Patients	277 (100)
Tumour size (mm)	
Mean (+/- SD)	26 (+/- 18.05)
Median (Range, mm)	22 (1-110)
Papillary	236 (85%)
Follicular	41 (15%)
Multi-focal	89 (32%)
Node Positive	56 (20%)
Distant Metastatic Disease	8 (3%)
Lymphovascular Invasion	44 (16%)
Personal History of Other Cancer[1]	25 (9%)
Family History of DTC[2]	23 (8%)

1: Excluding non-melanomatous skin cancer
2: First degree relatives only.

Table 8: Clinico-pathological patient and tumour characteristics

The minor allele frequency was higher in patients with DTC (0.45) compared to controls (0.34) and conferred an increased risk for DTC (per allele OR 1.61, 95% CI1.27-2.04, p=0.00008) (Table 9). Both variant genotypes had an increased risk for DTC and demonstrated an allele-dosage association; heterozygous genotypes (AG) had an odds ratio of 1.66 (95% CI1.16-2.39, p=0.00555) when compared with wild-type states (GG), increasing to 2.93 (95% CI1.70-5.05, p=0.00007) for rare homozygotes (AA).

rs965513 (G→A)	Common Homozygote (GG), n(%)	Heterozygote (AG), n(%)	Rare Homozygote (AA), n(%)	Minor Allele Frequencies
Cancers:	77 (28%)	151 (55%)	49 (17%)	0.45
Controls:	129 (42%)	152 (49%)	28 (9%)	0.34
Odds Ratio (95% CI)		1.66 (1.16-2.39)	2.93 (1.7-5.05)	
Significance (p)		0.00555	0.00007	

Table 9: Genotype frequencies, genotypic odds ratios, and minor allele frequencies in DTC for all cases vs controls

Subgroup analysis demonstrated statistically significant associations between the both variant genotypes (AG, AA) and DTC, for female gender but not for males. DTC risk association was also present for rare homozygotes, rather than heterozygous states, for tumours >10 mm, but not for patients with sub-centimetre DTC (Figures 26, 27 & Table 11).

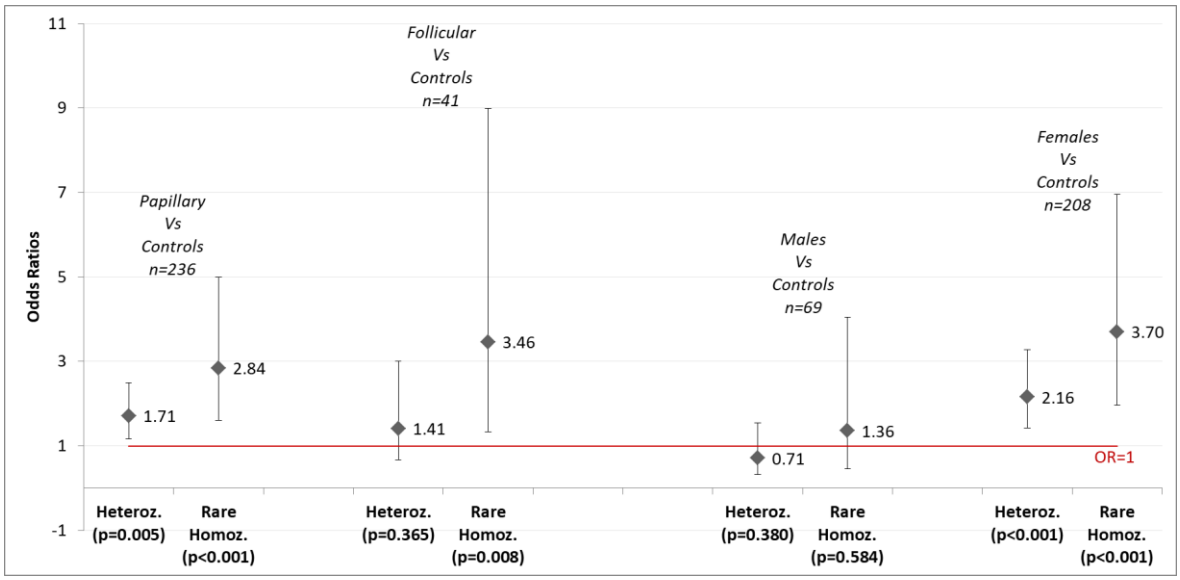


Figure 26: Genotypic odds ratios for sub-groups vs controls comparing rare homozygotes and heterozygotes with wild-types states: histological subtypes and gender

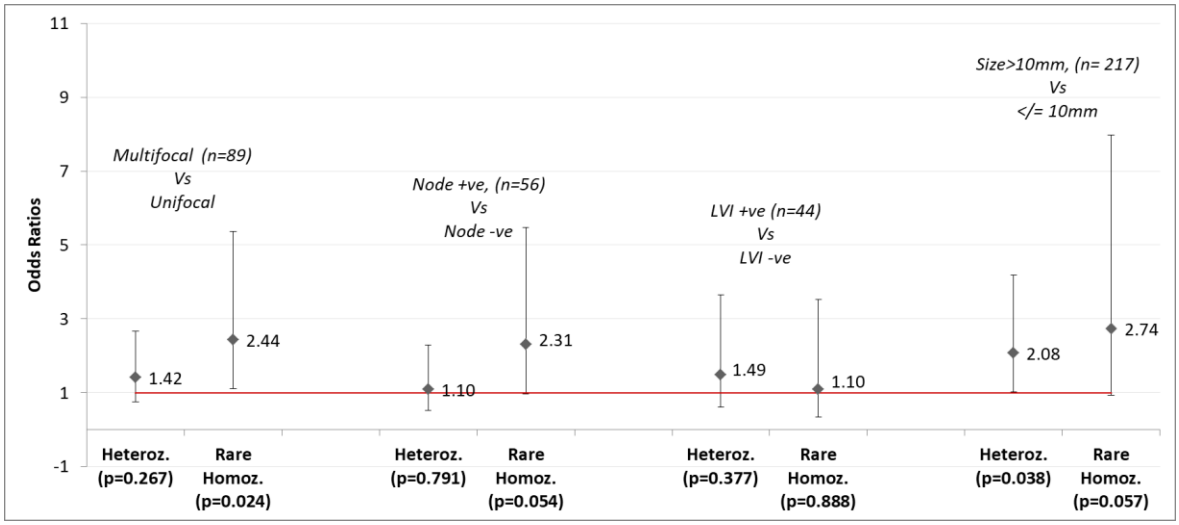


Figure 27: Genotypic odds ratios comparing rare homozygotes and heterozygotes with wild-types states for multi-focality, nodal status, lymphovascular invasion status and tumour size

Variations in MAF and genotypic odds ratios were also observed between Irish and French subgroups, with the minor allele observed more frequently among patients recruited in Ireland, in addition to conferring a greater risk for DTC compared with the French cohort (Table 10). In addition to higher genotypic odds ratios among the Irish subgroup, an increased per-allele ratio of 1.84 (95% CI 1.40-2.41, p=0.00001) was also observed, compared with a per-allele odds ratio of 1.31 among the French subgroup (95% CI 0.95-1.80, p= 0.09678).

rs965513 (G→A)	Common Homozygote (GG), n(%)	Heterozygote (AG), n(%)	Rare Homozygote (AA), n(%)	Minor Allele Frequencies
Controls:	**129 (42%)**	**152 (49%)**	**28 (9%)**	**0.34**
Irish Cases:	**39 (23%)**	**96 (57%)**	**33 (20%)**	**0.48**
Odds Ratio (95% CI)		2.09 (1.35-3.24)	3.898 (2.10-7.23)	
Significance (p)		0.00093	<0.00001	
French Cases:	**38 (35%)**	**55 (50%)**	**16 (15%)**	**0.40**
Odds Ratio (95% CI)		1.228 (0.76-1.98)	1.94 (0.95-3.96)	
Significance (p)		0.39606	0.06571	

Table 10: Genotype frequencies, genotypic odds ratios, and minor allele frequencies in DTC for French and Irish populations vs controls

SNP G>A	Wild-type (GG)	Heterozygote (AG)	Rare Homozygote (AA)	Minor allele
All Cases	77	151	49	0.45
All Controls	129	152	28	0.34
Odds Ratio (CI)		1.66 (95% CI 1.16-2.39)	2.93 (95% CI 1.7-5.05)	
p-value		0.00555276	0.00007621	
Papillary Cases	65	131	40	0.48
All Controls	129	152	28	0.34
Odds Ratio (CI)		1.71 (95% CI 1.17-2.5)	2.84 (95% CI 1.61-5.0)	
p-value		0.00531210	0.00024632	
Follicular Cases	12	20	9	0.46
All Controls	129	152	28	0.34
Odds Ratio (CI)		1.41 (95% CI 0.67-3.0)	3.46 (95% CI 1.33-8.99)	
p-value		0.36518017	0.00797090	
Male Cases	26	30	13	0.41
Male Controls	19	31	7	0.39
Odds Ratio (CI)		0.71 (95% CI 0.33-1.54)	1.36 (95% CI 0.46-4.05)	
p-value		0.38021705	0.58388242	
Female Cases	51	121	36	0.46
Female Controls	110	121	21	0.32
Odds Ratio (CI)		2.16 (95% CI 1.42-3.27)	3.7 (95% CI 1.96-6.96)	
p-value		0.00026969	0.00003028	
Multi-Focal Cases	19	49	21	0.51
Unifocal Cases	53	96	24	0.42
Odds Ratio (CI)		1.42 (95% CI 0.76-2.67)	2.44 (95% CI 1.11-5.36)	
p-value		0.26740704	0.02448424	
Node +ve Cases	13	28	15	0.52
Node -ve cases	60	117	30	0.43
Odds Ratio (CI)		1.1 (95% CI 0.53-2.29)	2.31 (95%CI 0.97-5.47)	
p-value		0.79133678	0.05408739	
LVI Cases	9	28	7	0.48
Non LVI Cases	24	50	17	0.46
Odds Ratio (CI)		1.49 (95% CI 0.61-3.65)	1.1 (95% CI 0.34-3.53)	
p-value		0.37714114	0.88753708	
>10mm Cases	54	124	39	0.47
</=10mm Cases	19	21	5	0.34
Odds Ratio (CI)		2.08	2.74	
p-value		0.03766692	0.05708887	

Table 11: Subgroup analysis: genotypic odds ratios and minor allele frequencies

4.4 Discussion

Molecular biomarkers have the potential to improve our estimation of DTC risk, and possess a number of advantages over conventional biomarkers. In particular, germline mutations remain stable irrespective of patient and disease factors or treatment, are measurable at or before disease onset, and are amenable to high-throughput assays, now commonly available in modern laboratories. In light of DTC-specific 5 year survival rates in excess of 95%, combined with increasing rates of DTC incidence worldwide, the challenge for clinicians is to avoid over-diagnosing and over-treating potentially low grade and indolent thyroid lesions, while appropriately managing those that may require more aggressive treatment. Our results demonstrate an association between one such biomarker, the rs965513 SNP in the FOXE1 gene, and an increased risk for development of DTC. Furthermore, we demonstrated an association with disease phenotype and therefore, this marker may confer prognostic benefits to improve DTC risk stratification.

The rs965513 SNP was initially identified as a risk factor for DTC (OR = 1.75, $p<0.001$) in an Icelandic genome-wide association study by Gudmundsson et al in 2009.[212] Since then, a number of studies have examined the association in various populations, including two large meta-analyses, which both supported an association between rs965513 and DTC susceptibility, but also reported widespread heterogeneity between study methodologies and outcomes, in particular among studies examining caucasian populations. [213,215] Furthermore, a number of studies have also reported an absence of association between DTC and the risk allele.[216,217]

To date, just one study has examined the association between rs965513 and DTC susceptibility in a UK population, while no published work has included data from Irish or French cohorts.[138] Incidence of thyroid cancer varies considerably between populations. Amongst European nations, the highest rates of thyroid cancer are seen in Lithuania (15.5/100,000), Italy (13.5/100,000) and France (11.7/100,000).[218] The incidence in Ireland is reported as 5.8/100,000 in 2017.[219] Furthermore, the rate of change in incidence also varies geographically; for example in France incidence has risen from 3.4 to 11.7 per 100,000 from 1983 to 2002, while in Sweden incidence has remained more stable, reported as 2.4/100,000 in 1958 and 3.5/100,000 in 2002.[220] In Ireland, incidence has increased three-fold, from 1.9/100,000 in 1994 to 5.8/100,000 in 2017.[3,219] With such significant geographical variation in thyroid cancer epidemiology, and reported population-based heterogeneity in DTC risk associated with rs965513, further validation of this risk allele in multiple cohorts is warranted.

An allele dose association between the rs965513 variant allele and DTC was demonstrated in this study, with odds ratios of 1.66 and 2.93 for heterozygous and rare homozygous states respectively and a per allele odds ratio of 1.61 compared to controls. This compares to per-allele odds ratios of

1.99, 1.75 and 1.82 reported in UK (n=781), Icelandic (n=192) and Polish (n=1795) cohorts respectively, while one Portuguese study reported a per allele odds ratio as high as 2.81 in a group of 80 patients with sporadic non-medullary thyroid cancer.[138,139,212,221] The frequency of the minor allele was 0.45 in our cohort which compares to 0.49, 0.49, 0.46 and 0.61 in the UK, Icelandic, Polish and Portuguese cohorts respectively. Variation in reported DTC between populations and among subgroups may arise for various reasons including true biological differences, variability in exposure to modifiable environmental risk factors or differences in sample size and study methodology.

This study recruited patients from tertiary centres in France (n=109) and Ireland (n=168). The risk allele was more prevalent in the Irish than the French subgroup (MAF Irish 0.48, French 0.4 and Control group 0.34), with associated higher per-allele odds ratios evident in the Irish subgroup, compared with the French (OR=1.31, p=0.096 vs OR=1.84, p=0.00001 respectively). Presence of the rare homozygous genotype among the French subgroup conferred an odds ratio for DTC of 1.94 (p=0.066) in France, however, for rare homozygous genotypes in the Irish subgroup, this rose to an odds ratio of 3.9 (p<0.00001). An almost four fold increase in DTC susceptibility in the presence of two variant alleles at the locus represents a significant risk factor for DTC, warranting consideration for its inclusion as a prognosticator for DTC. While our results suggests its impact on DTC risk may be more significant in Irish rather than French patients, it is notable that only 15% (n=16/109) of French subjects were rare homozygous carriers; assessment of a larger French cohort may also yield a statistically significant odds ratio for DTC. Furthermore, a limitation of this study is the lack of French subjects included in the control arm, which may limit conclusions that can be drawn from subgroup analysis specific to the French subgroup.

The minor allele frequency measured 0.46 in female cases and 0.32 in female controls, compared to a narrower differential between male cases and controls of 0.41 and 0.39 respectively. Our cohort exhibited an allele dose gender association, with female risk genotypes carrying a higher risk for DTC compared to female controls, while no significant association was demonstrated between male variant genotypes vs male controls. Heterozygous females carried an odds ratio of 2.6 (95% CI 1.42-3.27, p=0.0003), rising to 3.7 (95% CI 1.96-6.96, p=0.00003) for rare homozygous females. In contrast, equivalent odds ratios for male variant genotypes vs controls measured 0.71 and 1.36 (p=ns) for heterozygotes and rare homozygous carriers respectively. As expected, the gender distribution of DTC gives rise to insufficient power to adequately assess the variant effect in our male cases (n=69, 25%). However, given the gender differential in MAF, in addition to significant odds ratios observed for female genotypes, there is certainly a gender variance evident. Of the above mentioned studies, Liyanarachchi et al comment explicitly on gender specific risk conferred by the

variant; of their 1,795 polish DTC cases, no difference in MAF or DTC risk was evident between genders. Similarly, Gudmundsson et al reported an absence of significant MAF or rs965513 risk difference between genders in their Icelandic cohort.

In addition to contributing to risk estimation for the initial development of DTC, germline SNPs identified by GWAS or candidate gene studies may also impact the clinical course of DTC after diagnosis, and estimate risk for the development of particular pathological characteristics. Our data suggests that rs965513 is associated with tumours >10mm and tumour multi-focality, while a recent review by Jendrzejewski et al also identifies rs965513 as being associated with increased DTC tumour size, but also extra-thyroidal extension.[211] Other germline mutations have also been linked to additional pathological characteristics including nodal disease burden, metastatic disease and disease-specific mortality.[211] It follows that testing for rs965513 as part of a multi-gene mutational panel may not only estimate the likelihood of DTC occurrence, but also aid in stratifying patients in terms of locoregional, metastatic or recurrent disease risk, thereby informing decisions around treatment strategies such as the need for completion thyroidectomy, nodal dissection, radioactive iodine or TSH suppression. Furthermore, genotyping patients for rs965513 potentially adds valuable information for the management of cases that fall into an indeterminate or "individualised decision making" group as described in the British Thyroid Association guidelines, which are currently based on standard clinicopathologic features.[52]

4.5 Conclusion

Our data is concordant with findings of published GWAS and meta-analyses confirming that rs965513 is a low penetrance variant associated with DTC susceptibility. We conclude that assessment of rs965513 should be considered as part of a gene mutation panel to improve DTC risk stratification in patients with this common malignancy.

5 Chapter 5 - The impact of heritable miRNA-associated polymorphisms on differentiated thyroid cancer risk

5.1 Introduction

Micro RNAs (miRNAs) are short non-coding RNAs, measuring 21-24 nucleotides in length, which exert post-transcriptional effects on gene expression. MiRNAs act as gene regulators in animals, typically by binding to complimentary sites in the 3' untranslated region (UTR) of mRNA, forming a complex known as a miRNA-induced silencing complex (miRISC), which results in silencing or inhibition of protein translation from the target mRNA (Figure 16).[151] MiRNAs have been shown to be involved in almost all aspects of normal human biology including stem cell differentiation, haematopoiesis, apoptosis, cardiac and skeletal muscle development, neurogenesis, insulin secretion, cholesterol metabolism and the immune response.[151]

While miRNAs play an integral role in normal cell function, dysregulation may be associated with disease. MiRNAs have been demonstrated to regulate cell differentiation and proliferation; failure of these processes to be regulated may result in carcinogenesis, with many cancerous tissues expressing miRNAs at a lower level than in equivalent healthy tissue.[151] Oncogenesis in the setting of reduced miRNA activity suggests a tumour suppressor role. Similarly, up-regulation of various miRNAs associated with carcinogenesis has also been described, with these miRNA being described as oncomiRs. The first disease state to be associated with miRNA in humans was chronic lymphocytic leukaemia.[153] Many cancers, including differentiated thyroid cancer, have subsequently been linked with miRNA function and dysfunction.[154,155]

MiRNA dysregulation has been implicated in differentiated thyroid cancer (DTC) carcinogenesis.[159] Several works have assessed miRNA in DTC with a subset being commonly dysregulated in a number of studies across different populations; these include miR-146a, miR-146b, miR-222, miR-221 and miR-181b.[27,159,160]

MiRNA expression and function can be affected by the occurrence of single nucleotide polymorphisms (SNPs). Given the mechanism of miRNA/mRNA interaction, this may result from the presence of a SNP in the seed miRNA gene sequence (miR-SNP) or within a 3' UTR target site of the miRNA (miR-TS-SNP), both with the potential to affect the expression of microRNA targets.[156] Furthermore, increasing numbers of SNPs have been reported in various stages of miRNA biogenesis, including pri-, pre- and mature miRNA sequences (Figure 16).[156] Given that miRNA can have hundreds of binding sites, it follows that miR-SNPs have the potential for more significant biological

effects than miR-TS-SNPs.[157] MiRNA sequences are very short and have been shown to be relatively well conserved, which contrasts with the large numbers of less conserved non-coding 3' UTRs in the human genome, indicating that the frequency of binding site SNPs is much higher that miR-SNPs, with each miR-TS-SNP exhibiting more discrete effects compared to miR-SNPs; this characteristic is more desirable for genotype-phenotype, case-control studies.[158]

A single nucleotide variant (rs2910164) in the precursor stem region of pre-mir-146a is thought to reduce the stability of the pri-miR and affect processing of pri- to pre-miRNA; thus impacting expression of mature miR-146a.[222] Presence of this polymorphism has been associated with varying degrees of increased risk for DTC, however, reports conflict as to its effect size and whether or not the association applies to heterozygous or homozygous genotypes, or both.[27,138,222,223]

Rs17084733 is a G>A polymorphism located in the region of the microRNAs miR-221 and miR-222 domain in the v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) 3' UTR. Presence of this SNP in DTC patients has been associated with upregulation of these associated miRNAs and subsequent down-regulation of the KIT transcript.[160] miR-221 and miR-222, are encoded in tandem on the X chromosome and regulate $p27^{Kip1}$, a key cell cycle regulatory protein. Overexpression of miR-221 and miR-222 has been reported in DTC across multiple studies, and is associated with DTC susceptibility, and also with adverse DTC prognostic features such as disease metastases and recurrence.[224-226]

Constitutive activation of the mitogen-activated protein kinase (MAPK) signalling pathway is a significant event in the genesis of many cancers; but it is particularly relevant to differentiated thyroid cancer, with somatic mutations in MAPK-associated genes reported in over 70% of DTC.[227,228] The Kirsten rat sarcoma (KRAS) mammalian viral oncogene plays a major role in the MAPK pathway (Figure 11). The rs61764370 T>G polymorphism affects the interaction of miRNA let-7a with the KRAS 3'-UTR binding site, thereby modulating RAS expression and has been implicated in breast, ovarian, lung, colorectal and bone cancers, among others.[229-233] Despite the significant role of KRAS in DTC risk, no study has examined the influence of the rs61764370, KRAS polymorphism, on DTC susceptibility in any population.

This study investigates the impact of selected miRNA-associated variants on susceptibility to differentiated thyroid cancer and phenotypes in a Western European population.

5.2 Methods and materials

5.2.1 Study populations

Two Western European cohorts were assessed for this candidate gene, case-control, analysis of miRNA-associated polymorphisms. Specimens and associated clinico-pathological data were collected from patients attending endocrine cancer clinics at tertiary referral centres in the West of Ireland (University Hospital Galway) and in the South of France (Assistance Publique Hôpitaux de Marseille). Samples were gathered as part of a collaborative multicentre study to establish a thyroid cancer biobank at the Discipline of Surgery in the Lambe Institute for Translational Research at University Hospital Galway. Patients with a confirmed histological confirmation of DTC were included. Exclusion criteria included those with benign thyroid disease, medullary or poorly differentiated thyroid cancers and those patients with known pathogenic syndromes or germline mutations in cancer predisposition genes. Controls comprised community volunteers, over the age of 60, without a personal or first degree family history of malignancy, excluding non-melanomatous skin cancer.

All patients and controls provided informed written consent prior to inclusion in the study. Ethics approval was granted by the local institutional Research Ethics Committee, at University Hospital Galway. Demographic and clinico-pathological data was recorded at the time of specimen collection from patient self-reporting, electronic histopathology database and patient records.

5.2.2 Target SNPs and reagents

Three variants were assessed as part of this study, with each polymorphism associated with either a miRNA precursor or a miRNA binding site. Two specimen collection methods were employed; either a 10 mL sample of EDTA stabilised whole blood or a buccal swab salivary sample collected using a DNA Genotek Oragene 575 collection kit. Applied Biosystems SNP Genotyping TaqMan assays, utilising sequence-specific probes and PCR primer pairs were used to detect each miRNA associated variant under investigation. Polymorphisms examined included the pre-mir-146a variant (rs2910164), the KIT variant (Rs17084733) and the KRAS variant (rs61764370); the TaqMan assays used are referenced as Assays C__15946974_10, C__34674348_10, and C__89129087_10 respectively (https://www.thermofisher.com/ie/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays.html).

5.2.3 Procedures

DNA was extracted from buccal swab and whole blood samples by manual ethanol precipitation. Following DNA extraction, evaluation of the DNA concentration and purity was performed with absorbance spectroscopy using the NanoDrop 1000 Spectrophotometer™. Samples with a 260:280 nm (A260/280) absorbance ratio ≈ 1.8 were accepted as pure DNA.[178]

DNA samples were initially diluted to a standardised DNA concentration, prior to real-time polymerase chain reaction (RT-PCR) to amplify the targeted DNA using sequence-specific primers, and assessment of polymorphism-specific genotype by allelic discrimination using the Applied Biosystems TaqMan platform. The TaqMan SNP-specific assay contains a VIC dye-labelled probe, a FAM dye-labelled probe, and two target-specific primers. The wild-type SNP allele and the mutant SNP allele are each targeted using these region specific forward and reverse primers. The TaqMan probes use fluorescent reporter dyes, VIC and FAM, which attach to the 5' end, with the complementary quencher dye attaching to the 3' end. Amplification is performed using a thermal cycling RT-PCR system. Throughout amplification, each probe binds to its associated allele of interest and the TaqMan polymerase enzyme cleaves the bound probe, generating a fluorescent signal which is interpreted to determine the genotype. Allelic discrimination plots are then generated, with fluorescence of FAM and VIC plotted on X and Y axes to visually demonstrate the three genotypes. Manual quality control of the automatic genotype calling is performed using multicomponent plots. All DNA genotyping experiments were undertaken using either the Applied Biosystems StepOnePlus Real-Time PCR System or the 7900HT Fast Real-Time PCR System.[180]

5.2.4 Data management and Statistical Analysis

Biobank data and inventory utilised for this study is managed using the Shire Genetic Patient Database Software, a secure database prospectively maintained at the Discipline of Surgery NUI Galway. MS Excel 2010 was used for raw data manipulation and IBM SPSS v22 for statistical analysis.

Parametric tests and means (± standard deviation) were utilised for normally distributed data while non-parametric tests and medians (range) were utilised for non-normally distributed data. Pearson's chi-squared tests were used to compare distributions of categorical variables, while one way ANOVA with Tukey post-hoc analysis was utilised for comparisons involving three or more categorical, independent samples.

A case–control methodology was utilised, comparing genotypic and allelic frequencies of the variant in patients with DTC, to frequencies in unaffected controls. Chi-squared tests were used for

case-control genetic association analysis. Hardy-Weinberg Equilibrium tests of biallelic SNPs were performed using the Pearson's chi-squared test and illustrated using De Finetti ternary plots.

5.3 Results

280 patients with histologically proven DTC provided samples for genotyping. 110 patients were recruited from a tertiary referral centre in the South of France, while 170 patients were recruited from a tertiary centre in the West of Ireland. Of this overall case cohort, genotyping was successfully undertaken in 175, 275 and 274 patients, for the miR146a (rs2910164, G>C), KIT (rs1708473, G>A) and KRAS (rs61764370, T>G) variants respectively, while 637, 440 and 669 cancer-free control subjects were also genotyped for miR146a, KIT and KRAS variants respectively. Clinical and pathological patient characteristics are outlined in Tables 12 and 13. All case and control cohorts were demonstrated to be in Hardy Weinberg equilibrium (X^2, $p>0.05$, Table 14, Figure 28).

n (%)	miR146a Cases	miR146a Controls	KIT Cases	KIT Controls	KRAS Cases	KRAS Controls
Total:	175 (100%)	637 (100%)	275 (100%)	440 (100%)	274 (100%)	669 (100%)
Gender - Female	131 (75%)	637 (100%)	205 (75%)	383 (87%)	205 (75%)	669 (100%)
Gender - Male	44 (25%)	0 (0%)	70 (25%)	57 (13%)	69 (25%)	0 (0%)
Ethnicity - European Caucasian:	161 (92%)	637 (100%)	252 (92%)	440 (100%)	251 (92%)	669 (100%)
Ethnicity - Other (Asian, African):	14 (8%)	0	23 (8%)	0 (0%)	23 (8%)	0 (0%)
Collection Site - France	40 (23%)	0	90 (33%)	0 (0%)	91 (33%)	0 (0%)
Collection Site - Ireland	121 (77%)	637 (100%)	162 (67%)	440 (100%)	160 (67%)	669 (100%)
Age - Mean (+/- SD)	45.9 Years (+/- SD 15.4)	70.2 years (+/- SD 6.7)	46.9 years (+/-SD 15.0)	70.7 years (+/- SD 6.7)	46.9 years (+/-SD 15.1)	75.5 years (+/- SD 8.0)
Age - Median (range)	43 years (16-84)	69 years (60-98)	45 years (16-84)	70 years (60-98)	45.5 years (16-84)	75 years (60-102)

Table 12: Patient and control cohort characteristics

n (%)	**All Cases**	**miR146a Cases**	**KIT Cases**	**KRAS Cases**
Total	280 (100%)	175 (100%)	275 (100%)	274 (100%)
Histology:				
Papillary	239 (85%)	154 (88%)	235 (85%)	233 (85%)
Follicular	41 (15%)	21 (12%)	40 (15%)	41 (15%)
Tumour Size:				
Mean (+/- SD, mm)	26.4 (+/-SD 18.1)	26.4 (+/-SD 18.4)	26.1 (+/-SD 18.2)	25.9 (+/-SD 18.0)
Median (Range, mm)	23 (1-110)	23 (1-110)	22 (1-110)	21 (1-110)
Tumour T Stage:				
T_1	127 (45%)	77 (44%)	126 (46%)	127 (46%)
T_2	80 (29%)	60 (34%)	78 (28%)	77 (28%)
T_3	58 (21%)	33 (19%)	56 (20%)	55 (20%)
T_4	1 (0%)	1 (1%)	1 (0%)	1 (0%)
T Unknown / Not assessed	14 (5%)	4 (2%)	14 (5%)	14 (5%)
Tumour N Stage:				
N_0	72 (26%)	39 (22%)	71 (26%)	70 (26%)
N_1	56 (20%)	39 (22%)	53 (19%)	53 (19%)
N_2	1 (0%)	1 (1%)	1 (0%)	1 (0%)
N_x	151 (54%)	96 (55%)	150 (55%)	150 (55%)
Metastases, M_1	10 (4%)	7 (4%)	8 (3%)	10 (4%)
Multi-focality	90 (32%)	58 (33%)	87 (32%)	89 (32%)
Extra-thyroidal extension	43 (15%)	25 (14%)	41 (25%)	42 (15%)

Table 13: DTC clinico-pathological features

	miR146a, G>C		KIT, G>A		KRAS, T>G	
Genotypes:	**Cases (n=175)**	**Controls (n=637)**	**Cases (n=275)**	**Controls (n=440)**	**Cases (n=274)**	**Controls (n=669)**
Wild type	95	429	221	322	232	569
Heterozygous	65	181	52	106	41	98
Rare homozygous	15	27	2	12	1	2
Alleles:						
Wild type allele	255	1039	494	750	505	1236
Mutant allele	95	235	56	130	43	102
Minor allele frequency (MAF)	0.27	0.18	0.54	0.61	0.08	0.08
Hardy Weinberg Equilibrium*	0.42	0.16	0.58	0.36	0.57	0.30

Table 14: Genotypic and allelic frequencies among case and control cohorts. *HWE demonstrated using Pearson's chi-square (df = 1). Loci assumed accepted be in HWE where p>0.05.

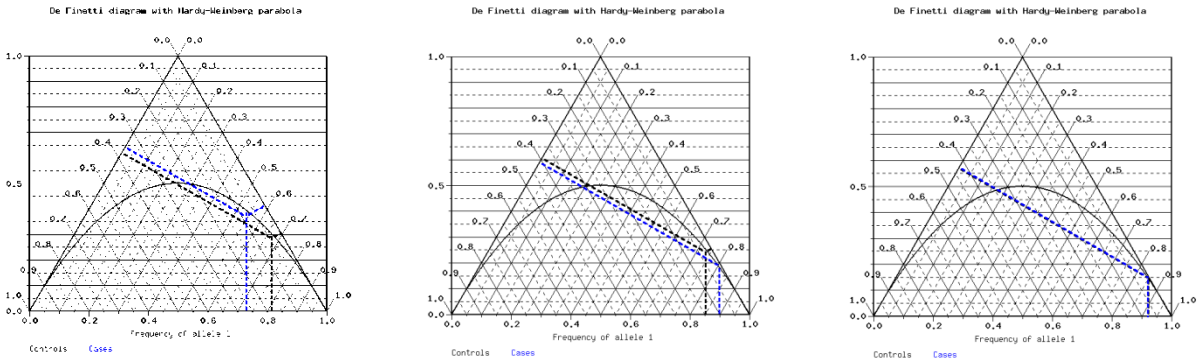


Figure 28: De Finetti ternary plots illustrating cases and controls in relation to the Hardy Weinberg Parabola for miR146a, KIT and KRAS genotypes, from left to right respectively.

Cases are represented by the blue dashed line, controls are represented by the black dashed line. Length of vertical line: frequency of genotype GA; length of left perpendicular line: frequency of genotype GG; length of right perpendicular line: frequency of genotype AA; Intersection of Hardy-Weinberg parabola and vertical line: frequency of genotype GA in case of perfect Hardy-Weinberg equilibrium.[214]

5.3.1 miR146a variant: rs2910164, G>C

With regard to the miR146a variant (rs2910164, G>C), the minor allele was more frequently observed in cases than controls (0.27 vs 0.18), with an overall per allele odds ratio (OR) of 1.64 (p<0.001) for DTC, when compared to controls. For those with one copy of the mutant allele at the locus, (CG vs GG), a genotypic OR of 1.62 (p=0.008) was observed, rising to 2.51 (p=0.006) for rare homozygous carriers (CC vs GG). The minor allele frequency (MAF) was also higher for all case subgroups assessed, when compared to controls (Table 16 & 17). Presence of the miR146a variant allele was associated with higher DTC risk for males (per allele OR=1.85, p=0.010) than females (per allele OR =1.58, p=004), for papillary (per allele OR 1.66, p<0.001) rather than follicular tumours (per allele OR 1.57, p=0.205), and for those ≥ 45 years of age (per allele OR 1.98, p<0.001) compared to those younger than 45 years (per allele OR 1.37, p=0.096) (Table 16). When association was examined by treatment location, the association remained significant for those managed in both France (per allele OR 1.68, p=0.027), and Ireland (per allele OR 1.63, p=0.002). Notably, rare homozygous carriers (CC) recruited in France were found to have a higher genotypic OR of 3.41 (p=0.008), in contrast to the risk conferred by the CC genotype among Irish-recruited cases of 2.13 (p=0.057) (Tables 15, 16 & 17).

5.3.2 KIT variant: rs17084733, G>A

Presence of the KIT variant SNP (Rs17084733, G>A) was found to confer a protective effect against DTC, with ORs measuring less than unity demonstrated across all subgroups. The MAF among all cases (n=275) was 0.54, compared to 0.61 among the control cohort (n=440). The overall per allele OR for DTC in the presence of this SNP was 0.65 (p=0.012). Genotypic ORs measured 0.72 (p=0.077) for heterozygous carriers, improving to 0.24 (p=0.046) for rare homozygous carriers (Table 15). In terms of subgroup analyses, statistically significant associations were demonstrated between presence of the variant and female (per allele OR 0.62, p=0.013), but not male gender (per allele OR 0.74, p=0.294); papillary (per allele OR 0.64, p=0.013) but not follicular subtypes (per allele OR 0.73, p=0.391); and among those ≥45 years of age (per allele OR 0.57, p=0.013), but not younger (per allele OR 0.75, p=0.174) (Table 16). Presence of the KIT variant allele also conferred a statistically significant decreased risk for DTC among those patients recruited in France (per allele OR 0.56, p=0.024), however, significance was not retained for those recruited in Ireland (per allele OR 0.71, p=0.088) (Table 17).

5.3.3 KRAS variant: rs61764370, T>G

Frequency of the KRAS variant (rs61764370, T>G) was examined in 943 samples (274 DTC cases, 669 controls). Distribution of the minor allele did not vary significantly between groups, with both having a MAF of 0.08. No association was found between presence of this mutant allele and DTC risk, either overall (per G allele OR 1.03, p=0.869), or on subgroup analysis (Tables 15, 16 & 17).

Odds Ratio (95% CI) p-value	**N, cases**	**MAF, cases**	**per allele**	**Heterozygous Vs Wild type**	**Rare Homozygous Vs Wild type**
miR146a (G>C)	175	0.27	*per C Allele* 1.64 (1.25-2.17) p<0.001	*CG vs GG* 1.62 (1.13-2.33) p=0.008	*CC vs GG* 2.51 (1.29-4.48) p=0.006
KIT (G>A)	275	0.54	*per A Allele* 0.65 (0.47-0.91) p=0.012	*AG vs GG* 0.72 (0.42-1.04) P=0.077	*AA vs GG* 0.24 (0.05-1.10) p=0.046
KRAS (T>G)	274	0.08	*per G Allele* 1.03 (0.71-1.49) p=0.869	*GT vs TT* 1.03 (0.69-1.52) p=0.898	*GG vs TT* 1.22 (0.11-13.59) p=0.868

Table 15: Overall genotypic and per allele odds ratios for DTC

Odds Ratio (95% CI) p-value	n Cases	MAF Cases	per allele OR	Heterozygous Vs Wild type	Rare Homozygous Vs Wild type
miR146a (G>C):					
Males	44	0.3	**1.85 (1.15-2.99), p=0.010**	**2.26 (1.19-4.27), p=0.010**	2.27 (0.64-8.09), p=0.194
Females	131	0.26	**1.58 (1.16-2.15), p=0.004**	1.44 (0.96-2.17), p=0.079	**2.58 (1.25-5.31), p=0.008**
Papillary	154	0.27	**1.66 (1.24-2.21), p<0.001**	**1.51(1.03-2.21), p=0.035**	**2.80 (1.43-5.50), p=0.002**
Follicular	21	0.26	1.57 (0.78-3.17), p=0.205	**2.61 (1.09-6.25), p=0.026**	0.74 (0.04-13.03), p=0.428
Age < 45 years	91	0.24	1.37 (0.94-1.98), p=0.096	1.25 (0.77-2.02), p=0.364	2.02 (0.84-4.86), p=0.109
Age ≥ 45 years	84	0.31	**1.98 (1.39-2.83), p<0.001**	**2.13 (1.32-3.46), p=0.002**	**3.18 (1.35-7.46), p=0.005**
KIT (G>A):					
Males	70	0.11	0.74 (0.43-1.29), p=0.294	0.77 (0.41-1.45), p=0.420	0.49 (0.06-3.83), p=0.486
Females	205	0.1	**0.62 (0.43-0.91), p=0.013**	0.70 (0.46-1.05), p=0.085	**0.16 (0.02-1.25), p=0.047**
Papillary	235	0.1	**0.64 (0.45-0.91), p=0.013**	0.69 (0.46-1.02), p=0.064	0.28 (0.06-1.28), p=0.080
Follicular	40	0.11	0.73 (0.36-1.50), p=0.391	0.88 (0.41-1.91), p=0.750	0.41 (0.02-7.08), p=0.283
Age < 45 years	131	0.11	0.75 (0.49-1.14), p=0.174	0.90 (0.57-1.43), p=0.663	0.12 (0.01-2.17), p=0.053
Age ≥ 45 years	144	0.09	**0.57 (0.37-0.89), p=0.013**	**0.56 (0.34-0.92), p=0.022**	0.45 (0.1-2.03), p=0.284
KRAS (T>G):					
Males	69	0.07	0.95 (0.48-1.86), p=0.874	0.98 (0.49-1.99), p=0.964	1.91 (0.09-40.34), p=0.649
Females	205	0.08	1.06 (0.70-1.60), p=0.778	1.04 (0.67-1.61), p=0.859	1.65 (0.15-18.25), p=0.682
Papillary	233	0.09	1.14 (0.78-1.67), p=0.507	1.14 (0.76-1.71), p=0.537	1.47 (0.13-16.26), p=0.754
Follicular	41	0.04	0.46 (0.14-1.48), p=0.183	0.46 (0.14-1.51), p=0.190	2.96 (0.14-62.71), p=0.715
Age < 45 years	131	0.08	1.06 (0.65-1.72), p=0.828	1.10 (0.66-1.85), p=0.694	1.03 (0.05-21.61), p=0.534
Age ≥ 45 years	143	0.08	1.01 (0.63-1.63), p=0.968	0.95 (0.57-1.60), p=0.852	2.33 (0.21-25.92), p=0.478

Table 16: Minor allele frequencies and genotypic odds ratios for gender, histological and age subgroups compared to controls

Odds Ratio (95% CI) p-value	**n cases**	**MAF cases**	**per allele OR**	**Heterozygous Vs Wild type**	**Rare Homozygous Vs Wild type**
miR146a (G>C):					
Irish	126	0.27	**1.63 (1.10-2.23), p=0.002**	**1.77 (1.18-2.65), p=0.005**	2.13 (0.96-4.74), p=0.057
French	49	0.28	1.68 (1.06-2.68), p=0.027	1.27 (0.66-2.43), p=0.471	**3.41 (1.20-8.93), p=0.008**
KIT (G>A):					
Irish	168	0.11	0.71 (0.48-1.05), p=0.088	0.75 (0.49-1.17), p=0.207	0.40 (0.09-1.82), p=0.224
French	107	0.09	**0.56 (0.34-0.93), p=0.024**	0.66 (0.38-1.13), p=0.125	0.15 (0.01-2.48), p=0.071
KRAS (T>G):					
Irish	166	0.07	0.90 (0.56-1.44), p=0.667	0.93 (0.57-1.52), p=0.784	0.79 (0.04-16.62), p=0.479
French	108	0.09	1.24 (0.75-2.04), p=0.407	1.17 (0.68-2.04), p=0.567	3.20 (0.29-35.62), p=0.318

Table 17: Minor allele frequencies and genotypic odds ratios per tertiary treatment centre, West of Ireland vs South of France.

Odds Ratio (95% CI) p-value	n Cases	MAF Cases	n Controls	MAF Controls	per allele OR	Heterozygous Vs Wild type	Rare Homozygous Vs Wild type
miR146a (G>C):							
Multifocal vs Unifocal	58	0.25	112	0.27	0.89 (0.53-1.49), p=0.658	0.6 (0.299-1.202), 0.147	1.42 (0.44-4.57), p=0.555
Node positive vs Node Negative	40	0.25	135	0.28	0.87 (0.49-1.53), p=0.624	0.67 (0.31-1.46), p=0.312	1.08 (0.31-3.70), p=0.908
LVI vs No LVI	32	0.38	143	0.25	**1.82 (1.03-3.22), p=0.039**	**2.26 (0.99-5.17), p=0.050)**	2.52 (0.69-9.18), p=0.152
Size >10mm vs ≤10mm	116	0.25	27	0.22	1.19 (0.59-2.42), p=0.623	1.99 (0.73-5.40), p=0.174	0.74 (0.18-3.07), p=0.676
KIT (G>A):							
Multifocal vs Unifocal	87	0.10	188	0.10	0.94 (0.51-1.71), p=0.828	1.05 (0.55-2.00), p=0.887	0.43 (0.02-9.07), p=0.337
Node positive vs Node Negative	33	0.08	155	0.11	0.67 (0.25-1.77), p=0.412	0.73 (0.26-2.06), p=0.552	0.87 (0.04-18.55), p=0.500
LVI vs No LVI	45	0.11	230	0.10	1.13 (0.55-2.32), p=0.750	1.27 (0.58-2.76), p=0.553	1.05 (0.05-22.35), p=0.540
Size >10mm vs ≤10mm	231	0.11	44	0.06	0.21 (0. 08-5.32), p=0.128	2.01 (0.75-5.39), p=0.156	1.08 (0.05-22.98), p=0.500
KRAS (T>G):							
Multifocal vs Unifocal	89	0.08	184	0.08	0.97 (0.47-1.98), p=0.924	6.22 (0.25-154.46), p=0.151	1.12 (0.58-2.15), p=0.740
Node positive vs Node Negative	54	0.10	220	0.07	1.61 (0.75-3.47), p=0.219	1.45 (0.06-36.25), p=0.634	1.45 (0.70-2.97), p=0.313
LVI vs No LVI	44	0.05	230	0.08	0.52 (0.18-1.54), p=0.230	1.58 (0.06-39.59), p=0.648	0.51 (0.18-1.48), p=0.209
Size >10mm vs ≤10mm	230	0.08	44	0.08	0.92 (0.38-2.24), p=0.857	0.58 (0.02-14.40), p=0.663	0.98 (0.42-2.28), p=0.967

Table 18: Pathological subgroup analysis of minor allele frequencies and genotypic odds ratios

5.4 Discussion

MiRNA modulation plays an important role in thyroid tumourigenesis. Disruption of normal miRNA function exerts a pro-oncogenic effect; over-expression of a given miRNA may inhibit protein translation of a tumour-suppressor gene, whereas down-regulation of a different miRNA may increase levels of an oncogenic protein.[234] This work investigates the association of three polymorphisms which disrupt normal miRNA function in patients with DTC.

The miR146a family of miRNAs plays a role in inflammation, immune function, epithelial cell homeostasis and inhibition of invasion and metastasis, making them prospective candidates as cancer susceptibility genes. Dysregulation of miR146a has been implicated in many malignant and non-malignant inflammatory conditions including hepatocellular and gastric cancers, coronary artery

disease, inflammatory bowel disease and multiple sclerosis.[235-240] MiR146a and miR146b have also been shown to be upregulated and associated with adverse prognostic features, progression or invasion in papillary, follicular and anaplastic thyroid cancers.[241-243] However, some studies have demonstrated no deleterious association between miR146a and DTC risk; Zhang et al examined 1,238 PTC patients and 1,275 controls from northern China and found no association between rs2910164 and PTC risk, while Wei and colleagues also concluded that rs2910164 was not associated with increased risk of PTC or benign thyroid disease in a Chinese Han cohort of 753 PTC cases, 484 benign tumour cases and 760 controls, but may play a role in the transformation from benign tumour to PTC.[223,244] A 2018 meta-analysis from Chen et al including 3,993 DTC cases from eight studies also concluded that the variant had no effect on susceptibility to PTC, however, their subgroup analysis of Caucasians alone yielded a p-value approaching significance (p=0.062), which the authors comment may be indicative of an association, meriting additional study.[245] Our data supports a potential association between the rs2910164 variant and DTC risk in this western European patient cohort, with the minor allele evident in 27% of cases (vs 18% of controls), and an associated per allele OR of 1.64 (p<0.001). Incorporation of these new data into a further meta-analysis may strengthen the supposition that the variant is associated with development of DTC, particularly among Caucasians.

Our data demonstrates a per allele dosage effect for DTC risk, with heterozygous carriers exhibiting an odds ratio of 1.62 (p=0.008), which increases to 2.51 (p=0.006) for rare homozygous carriers. Jazdzewski and co-workers genotyped 608 PTC patients and 901 controls and found that GC heterozygous carriers exhibited a comparable associated increased risk for PTC (OR= 1.62, P=0.000007), but interestingly both wild type and rare homozygous states were protective against DTC (CC: OR=0.42, p=0.003; GG: OR=0.69, p=0.0006). They subsequently postulated that the lack of association between the rare homozygous rs2910164 genotype and disease was attributable to differential production of mature miRNA in tumours, with heterozygotes producing three alternate isoforms of the miRNA (miR-146a from leading strand, and miR-146a*G and miR-146a*C from passenger strand), each with its distinct set of target genes. This mechanism may provide an explanation for the heterogeneous outcomes seen across previous studies.[222,246]

Variation in outcomes between studies may also be linked to frequency of the variant among patient populations and ethnicities. Jazdzewski and coworkers included samples from three ethnically distinct populations (Finland, Poland and USA); with an approximately equal representation of cases from each cohort, and over half of the controls being Polish.[222] We demonstrated a MAF of 0.28 among French cases and 0.27 among our Irish cohort. These are comparable to the MAF reported in

Finnish cases (0.27) but higher than the MAF reported in Polish (0.22) or UK (0.24) cohorts, and lower than MAFs reported in American (0.30) and Chinese patients (0.57).[138,222] We require a cohort of French controls for comparative analyses before any conclusion can be made regarding the significance of this variant in the French population, given this evident variability in frequency of the SNP seen across different populations. This high MAF in DTC cases merits further investigation in a larger study with controls of matched ethnicity. Inter-population heterogeneity of the variant is exemplified by the MAF demonstrated in our cohort compared to our nearest geographical neighbours in the United Kingdom, where the MAF reported by Jones et al, in both control and case cohorts, was 0.24. The UK study therefore did not support an association of the variant with disease. Our criteria for inclusion of participants as controls was much more stringent than in the UK study, where controls were recruited from the national blood donor service, the 1958 Birth cohort, and from a separate colorectal cancer susceptibility gene discovery project (COloRectal Gene Identification study (CORGI)).

Gender has previously been examined as a factor which may influence the impact of the miR146a polymorphism on disease risk, postulated to arise due to the regulation of miRNA-146a expression by oestrogen in immune cells.[247,248] Our data demonstrates a MAF of 0.3 among males, and 0.26 among females. A greater increased risk for DTC was seen among males (per allele OR=1.85, p=0.010) compared to females (per allele OR=1.58, p=0.004), however, a notable limitation of this study is the small number of male controls genotyped across the three variants, limiting the conclusions that can be drawn from gender specific sub group analysis.

We also assessed the impact of the miR146a polymorphism on DTC phenotype and pathological characteristics. Our data suggests that presence of the variant is associated with an increased risk for lymphovascular invasion (LVI) in DTC with an observed genotypic odds ratio of 1.82 (1.03-3.22), p=0.039 (Table 18). The presence of LVI among DTC patients is independently associated with higher risk and poorer survival, and merits consideration for aggressive adjuvant therapy such as RRA.[249] The miR146a variant was not found to confer greater risk for multifocal disease, node positivity, or tumour size.

In addition to identifying up-regulation of mi146a in DTC, He et al also reported that overexpression of miR-221 and miR-222 was evident in ten patients with PTC, with resulting drastic down regulation of the KIT protein.[160] Of these ten patients, five were identified as heterozygotes for another germline single-nucleotide polymorphism, rs17084733, which impairs miR-221 binding and, consequently, KIT posttranscriptional regulation.[250] This G>A substitution is located within a seed region of the KIT 3'UTR at the miR-221/222 binding site, giving rise to a conformational change and a

resulting increase in free energy, indicating base pairing disruption in the complementary site.[251] While mature miRNAs measure up to 24 nucleotides long, their seed regions measure only 2 to 7 nucleotides in length and function to identify target mRNAs for binding.[251] The short length of the seed region suggests that even single nucleotide substitutions here, such as rs17084733, can significantly affect binding capacity of the miRNA and subsequent mRNA transcription, in this case resulting in down regulation of the KIT protein and an increased risk for DTC.[252] A limited number of studies have demonstrated an association between this polymorphism and other malignancies such as gastrointestinal stromal tumours and acral melanoma, while an absence of association was demonstrated in the setting of hepatocellular carcinoma. [252-254] This author could not identify any published studies validating the impact of rs17084733 on DTC risk.

Our data suggests that presence of the KIT variant rs17084733 is associated with a reduced risk for development of DTC. The variant allele was evident in 54% of cases (n=275) and 61% of controls (n=440), with an associated per allele odds ratio of 0.65 (p=0.012). A dosage allele effect was also evident with rare homozygous carriers exhibiting a lower genotypic OR compared to heterozygous carriers (AG: 0.72, p=0.077 vs AA: 0.24, p=0.046). While He and coworkers reported that 5 out of 10 PTC patients with over-expression of miR146a, miR221 and miR222 were heterozygous for the KIT variant SNP, they subsequently undertook a candidate gene case control analysis of 135 Finnish PTC cases and 94 controls, and found no association between the SNP and PTC risk. The MAF however, was observed less frequently among their cases (6.3%) compared to 9% and 11% for our French and Irish cohorts respectively. Our data suggests that presence of this variant is protective against PTC. For PTC alone, we calculate an odds ratio of 0.69 (p=0.064) for heterozygous carriers, falling to 0.28 (p=0.080) for rare homozygotes, while no significant association was demonstrated for follicular subtypes (per allele OR 0.73, p=0.391). We also demonstrated significance of the association among French DTC cases (per allele OR=0.56, p=0.024), but not Irish cases (per allele OR=0.71, p=0.088). This variation in outcomes between Finnish, French and Irish cohorts suggests varying effect size across populations warranting further evaluation in different populations. Finally, a significant association also remained evident for females (per allele OR=0.62, p=0.013), but not males (per allele OR=0.74, p=0.294) although this may be due to the expected/natural predominance of female cases in our cohort (n=205/275, 75%), and the resulting smaller sample size of male cases. The KIT variant rs17084733 was not found to confer an increased risk for DTC multifocality, lymphovascular invasion, node positivity, or tumour size.

We also investigated the impact of a germline T>G polymorphism (rs61764370) located in the 3' untranslated region (UTR) of Kirsten rat sarcoma viral oncogene homolog (KRAS), which disrupts

miRNA let-7 binding at that site. KRAS activating mutations have been associated with carcinogenesis and poor outcomes for many cancers, with evidence suggesting that the presence of the KRAS-variant rs61764370 disrupts binding of let-7, leading to a reduction in KRAS-negative regulation and initiating gain of function.[229,255-262] Somatic RAS mutations are found in all epithelial thyroid malignancies and KRAS plays a major role in the MAPK signalling pathway, activation of which is known to be significant event in DTC carcinogenesis.[113,263] It follows that the KRAS variant rs61764370 represents a potential biomarker for DTC. Following genotyping of 274 cases and 669 controls for the KRAS variant, a minor allele frequency of 8% was identified in both cohorts, with no significant association demonstrated between presence of the variant allele and DTC (per allele OR=1.03, p=0.869). This lack of association was sustained for heterozygous and rare homozygous genotypes, and also when subgroups including gender, age<45 years old, histological subtype, country of recruitment, DTC multifocality, lymphovascular invasion, node positivity and tumour size were assessed separately (Tables 16-18). Although disruption of the tumour suppressor let-7 by the KRAS-variant might be expected to contribute to DTC risk, Crowley et al also found no effect on KRAS gene expression or downstream PI3K or MAPK signalling following their in vitro examination of colorectal cancer cell lines with targeted knock-in of the polymorphism rs61764370. They postulate that when let-7 is impaired from binding, it becomes vulnerable to degradation causing an alteration in the transcriptional and miRNA profile of the cells, negating the effect on KRAS gene expression, which may be an explanation for the lack of association with DTC risk in our cohort. [264]

The significant advances in technology to investigate human genetic variation over recent decades make it unlikely that science will identify any further high penetrance, high risk, monogenic mutations such as the BRCA or RET mutations associated with breast or medullary thyroid cancers. It is more likely that assessment for multiple low risk, low penetrance mutations in multiple genes will lead to improved risk estimation for many cancers. The miRBase miRNA registry lists 1,115 mature human miRNAs, with many being pursued as diagnostic, prognostic or therapeutic targets in the battle against cancer, representing a promising source of risk biomarkers for cancers such as DTC.[265]

5.5 Conclusion

This study highlights miRNA-associated polymorphisms which may contribute to a polygenic risk assessment for development or prognostication in patients with DTC. In particular, an allele dosage effect is seen with the miR146a and KIT polymorphisms with noteworthy odds ratios particularly evident for homozygous carriers of these mutations. We also report an absence of association between the KRAS variant and DTC susceptibility.

6 Chapter 6 - Discussion

The average yearly incidence of thyroid cancer in Ireland for the period from 2011 to 2015 increased by more than 300%, compared to the period from 1996 to 2000.[2] Similar significant increases in incidence have also been observed globally and although this may partly be attributable to intensified surveillance with improved sensitivity and availability of imaging modalities, it has nonetheless resulted in an increasing burden on patients and treating clinicians.[164] Differentiated thyroid cancer is an increasingly heterogeneous disease with a wide spectrum of phenotypes ranging from indolent to aggressive variants. In the setting of increasing rates of DTC diagnoses, it is critical that risk stratification approaches are sufficient to guide appropriate management. Current best practice guidelines inadequately delineate between DTC subgroups with divergent risk profiles, owing to a paucity of good quality evidence to support recommendations for optimal management among specific subgroups.[52] The key to improving risk stratification and prognostication for patients with DTC may lie in an improved understanding of the molecular mechanisms of thyroid tumourigenesis, including knowledge of low risk mutant alleles which contribute to DTC susceptibility. The data presented assesses the impact of equivocal guidance, particularly for patients with intermediate size 1-4cm tumours, on contemporary practice patterns. Additionally, we measure the effect of single nucleotide polymorphisms in genes affecting thyroid biology on patients with DTC, and investigate the role of these genotypes in modifying DTC risk and their association with disease phenotype, which may contribute to improved risk stratification. We also examine the impact of mutations in miRNA, or in miRNA-mRNA binding sites on DTC susceptibility. In order to facilitate both clinical and experimental aspects of this work, we established and populated a multicentre thyroid cancer biobank and complementary clinical database, as a tool for examining both genetic and clinico-pathological characteristics of differentiated thyroid cancer.

We assessed practice patterns in the management of 178 patients with DTC in an Irish tertiary referral centre, focusing on patients with intermediate size (1-4cm), low risk tumours where a paucity of high-level evidence prevents current guidelines from supporting a definitive therapeutic approach. While concordance with 2014 BTA guidelines was demonstrated in 94% of surgical decisions, we found that in cases where there was discordance with guidelines (n=10, 6%), there was a tendency towards over-treatment with nine of these undergoing total or completion thyroidectomy where lobectomy was recommended by BTA guidelines. Additionally, almost half of patients assessed, typically with intermediate-sized tumours without specified risk factors for recurrence, were subject to equivocal recommendations with respect to either surgery or RRA,

based on BTA guidelines, whereby a "personalised decision making" approach was suggested. Almost all of these patients, where there was a decision regarding the need for either completion thyroidectomy (n=32) or RRA (n=80), proceeded to the more aggressive treatment option of completion surgery or radioiodine therapy. The absence of sufficient evidence to support a definitive treatment course in this substantial cohort despite multiple large retrospective studies, suggests that any benefit gained by this strategy is likely to be small; the potential oncological benefits of these more aggressive options may be outweighed by both physical and psychological morbidity in many cases.[191]

Improved understanding of the genetic and molecular mechanisms underlying thyroid carcinogenesis is imperative to guide personalised treatment. Although the pathological mechanisms of thyroid cancer susceptibility are not yet fully understood, it remains unlikely that a significant high penetrance monogenic variant, such the RET proto-oncogene for MTC, remains undiscovered for DTC, however, detection of low penetrance alleles across multiple genes, while conferring small effect sizes individually, may cumulatively exert an overall polygenic effect approaching magnitudes more often associated with high-risk cancer syndromes and contribute to the estimation of risk. To this end, we investigated the impact of a polymorphism in the FOXE1 gene on DTC risk in a Western European cohort. This FOXE1 variant was initially identified in an Icelandic genome-wide association study in 2009, although never validated in an Irish or French population.[212] Given significant geographical variation in thyroid cancer epidemiology, and population-based heterogeneity in DTC risk associated with this variant, validation of this risk allele in multiple cohorts was merited.[218] We demonstrated a significant association between the FOXE1 variant and DTC susceptibility in our cohort. An allele dosage effect was observed with odds ratios of 1.66 and 2.93 for heterozygous and rare homozygous genotypes respectively, and a per allele odds ratio of 1.61 when compared to controls. Amongst Irish patients alone, presence of the rare homozygous genotype conferred an odds ratio of 3.9 ($p<0.00001$) for DTC risk. This almost four fold increase in DTC susceptibility in the presence of two variant alleles at the locus represents a significant risk factor for DTC. Furthermore, subgroup analysis suggests that rs965513 is associated with phenotypical characteristics such as tumour size >10mm (OR=2.74, p=0.05) and tumour multi-focality (OR=2.44, p=0.02) which may suggest its inclusion as a biomarker to improve risk stratification for patients diagnosed with DTC.

MiRNA dysregulation has also been implicated in differentiated thyroid cancer (DTC) carcinogenesis. Several works have assessed miRNA in DTC with a subset commonly dysregulated across different populations.[27,159,160] We sought to examine the impact of three germline mutations (miR146a, KIT &

KRAS variants) in miRNA, or in miRNA-mRNA binding sites, on DTC susceptibility. MiR146a is reported to be up-regulated and associated with adverse prognostic features in DTC, although the literature remains inconclusive, with heterogeneity between outcomes reported across multiple published works.[245] A recent meta-analysis concluded that the variant had no effect on susceptibility to PTC, however, their subgroup analysis of Caucasians alone yielded a p value approaching significance (p=0.062), which the authors comment may be indicative of an association, meriting additional study.[245] In this cohort, presence of the miR146a variant did exhibit a significant association with DTC risk. The minor allele was more frequently observed in cases than controls (0.27 vs 0.18), with an overall per allele odds ratio (OR) of 1.64 (p<0.001) for DTC, when compared to controls. For those with one copy of the mutant allele at the locus, (CG vs GG), a genotypic OR of 1.62 (p=0.008) was observed, rising to 2.51 (p=0.006) for rare homozygous carriers (CC vs GG). It is likely that a further meta-analysis incorporating this data may strengthen the supposition that the variant is associated with development of DTC, particularly among Caucasians. Presence of the miR146a variant was also found to be associated with lymphovascular invasion, a poor prognostic feature (genotypic OR=1.82 (p=0.039), associated with poorer survival in DTC.

A further mutation within the seed region of the KIT 3' UTR at the miR 221/222 binding site has been found to result in down regulation of KIT protein translation and has been postulated to increase risk for DTC, although only one study could be identified that examines the impact on a small DTC cohort, with no significant association found.[160] Our data suggests that presence of the KIT variant rs17084733 is associated with a reduced risk for development of DTC. The variant allele was evident in 54% of cases (n=275) and 61% of controls (n=440), with an associated per allele odds ratio of 0.65 (p=0.012). A dosage allele effect was also evident with rare homozygous carriers exhibiting a lower genotypic OR compared to heterozygous carriers (AG: 0.72, p=0.077 vs AA: 0.24, p=0.046).

We also investigated the impact of a novel germline T>G polymorphism in the 3' UTR of KRAS. RAS mutations are common in follicular derived thyroid cancers and KRAS plays a major role in the MAPK signalling pathway, activation of which is well known to be a significant event in DTC carcinogenesis.[229,256-263] We have demonstrated a minor allele frequency of 8% in both DTC cases and controls, with no significant association evident between presence of the KRAS mutant allele and DTC susceptibility (per allele OR=1.03, p=0.869).

6.1 Future Directions

It is clear from this work that further research will improve our knowledge of risk factors for the development of DTC and also for risk stratification for patients with DTC. Without improved risk assessment and prognostication tools, there remains insufficient evidence to inform clinical practitioners on the appropriateness of surgery and RRA for specific subgroups of patients. Further work should include efforts to elucidate other germline mutations which contribute to DTC risk; given the geographical heterogeneity of DTC risk, this would require further genome-wide association studies and subsequent candidate gene studies to be undertaken across multiple populations. With respect to germline mutations identified in this work as being risk factors for development of DTC and for poor prognostic features, it may be beneficial to include these SNPs as part of a risk profiling panel comprising multiple polymorphisms, to more accurately assess risk for DTC and the risk for poor outcomes as part of a prognostication tool. While similar multi-gene panels currently exist for assessment of somatic mutations in thyroid nodule FNA specimens, utilisation of these in combination with germline mutations may improve the accuracy and utility and allow for development of DTC risk assessment tools prior to DTC diagnosis.

With respect to the miRNA associated mutations studied in this work, it may be advantageous to assess the quantitative impact of these mutations on miRNA expression profiles within patient serum, normal thyroid parenchyma, tumour and tumour-associated normal thyroid tissues. Point mutations in pri- pre- or mature miRNA may influence the expression of mature miRNAs resulting in functional effects on cancer susceptibility and disease phenotypes. Examining the effect of such SNPs on circulating miRNA phenotypes may assist in further elucidating the impact of these mutations on the pathophysiology of DTC and potentially provide an alternative biomarker to estimate DTC risk and prognoses.

6.2 Conclusion

In conclusion, current best practice guidelines for the management of patients with DTC do not adequately delineate the appropriate management for specific subgroups of patients for which there is a paucity of high quality evidence to guide clinical practice. This work has demonstrated the extent to which some patients treated in tertiary referral centres are subject to equivocal guidelines due to the lack of evidence for these intermediate risk thyroid cancers. With a view to improving risk stratification for these patients, we have demonstrated a significant impact on overall DTC risk, disease phenotype and prognosis, associated with a germline mutation in the FOXE1 gene. Furthermore we have elucidated the effects of three miRNA-associated variants on DTC susceptibility, with the mir146a and KIT variants exhibiting a significant impact on DTC susceptibility, clinicopathological features and disease prognosis. Testing for these variants should be considered as part of a gene mutation panel to improve DTC risk estimation for patients with this common malignancy.

7 Chapter 7 - Appendices

7.1 Ethics approval

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Ospidéal Réigiúin Phairc na Muirlinne
Merlin Park Regional Hospital,
Galway, Ireland.

Tel: (091) 757 631

Research Ethics Committee
Unit 4
Merlin Park Hospital
Galway.

27th January, 2006.

Professor Michael Kerin
Department of Surgery
Clinical Science
University College Hospital
Galway.

Ref: 45/05 - The Provision of a Breast Cancer BioBank research resource for use in Molecular and Cellular Studies and Clinical Trials

Dear Michael,

The informed consent form for participation in the BioBank was approved by the CREC subject to a single amendment. It was felt that a stronger statement should be included to ensure that participants were aware their histological details would be linked to their clinical data and to their overall health overcome.

Yours sincerely,

Dr. S. T O'Keeffe
Chairman Research Ethics Committee.

Department of Surgery
School of Medicine
Clinical Science Institute
National University of Ireland, Galway
Ireland
09 December 2014

Dr. Shaun O'Keefe
Chairman, GUH Recognised Ethics Committee
Merlin Park, Unit 4
Galway

Re: Request for Amendment to protocol number 45/05 and C.A. 151, 'The provision of a breast cancer biobank research resource for use in molecular and cellular studies and clinical trials'

Dear Dr O'Keefe,

I am writing to the University Hospital Galway Research Ethics Committee to seek permission to make amendments to protocol 45/05 and C.A 151 such that tissue sampling may extend to include patients with cancers of thyroid origin. All tissue and clinical data collection and storage will mirror those already approved as set out in Protocols 45/05 and C.A. 151, including continued storage of all tissue in the Department of Surgery BioBank, NUI Galway.

No amendments to the patient consent or information documentation are required.

Unfortunately, we do not have a copy of the original Breast Cancer Biobank protocol, which was first submitted to the ethics committee approximately 23 years ago by Professor Fred Given. We have copies of all correspondence with the REC from the last 8 years, including copies of the Biobank Resource approval letters in this time. We hope you consider this amendment acceptable and thank you for taking the time to consider our request.

Details of these amendments are outlined in the attached CREC amendment form.

Yours sincerely,

_____
Professor MJ Kerin

Head of Department
Department of Surgery
School of Medicine
Clinical Science Institute
National University of Ireland, Galway

t: +353 91 544637
e: michael.kerin@nuigalway.ie

7.2 Patient information and consent

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

Patient Information

Introduction

We would like to invite you to participate in a clinical research initiative at Galway University Hospitals to establish a BioBank. The purpose of the BioBank is to set up a resource that can support a diverse range of research programmes intended to improve the prevention, diagnosis and treatment of cancer. You are under no obligation to take part and if, having read the information below, you would prefer not to participate, we will accept your decision without question.

Although major advances have been made in the management of cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research. Galway University Hospitals are actively involved in research that aims to identify markers that will predict how a cancer develops, progresses and responds to a variety of treatments. This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the diagnosis, treatment and outcome for those who have cancer. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

Your Involvement

If you volunteer to participate in our BioBank, there will be no additional risks to you outside those of your standard investigation and treatment. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Procedure

We invite all patients who are undergoing treatment and/or investigation to participate. All samples for research will be taken at the time you are attending the hospital for routine diagnostic tests.

(i) **Tissue Samples**

By participating, you give us consent to retain small pieces of your tissue obtained at the time of surgery. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland. This will not affect your diagnosis in any way.

(ii) **Blood Samples**

By participating, you give us consent to take an extra blood sample (equivalent of 4 teaspoonfuls) at the same time that your blood is being taken for routine tests. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland.

(iii) **Clinical Information**

By participating, you give us consent to store information relating to your diagnosis and treatment on a database. This information is only accessed by personnel directly involved in research within the Surgical Research Unit.

Further Information

If you would like further information about our BioBank, your participation and your rights, please contact the Surgical Research Unit (Tel: 091 524390).

If you would like further information about research projects that may be conducted, please contact your Consultant.

Thank you in anticipation of your assistance. Please read and sign the Consent section.

I have read the attached information sheet on the above project, dated _____ ☐

Please Initial Box

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. I understand that I may withdraw from the study at any time.

(Name of sponsor): ……

PARTICIPANT'S NAME: ……

CONTACT DETAILS: ……

PARTICIPANT'S SIGNATURE: ……

DATE: ……

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:

NAME OF CONSENTER, PARENT, OR GUARDIAN: ……

SIGNATURE: ……

RELATION TO PARTICIPANT: ……

DECLARATION OF INVESTIGATOR'S RESPONSIBILITY

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME OF RESEARCH NURSE OR INVESTIGATOR: ……

SIGNATURE: ……

DATE: ……

CONSULTANT: ……

Keep the original of this form in the investigators file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).

7.3 Questionnaire for participating patients

NUI Galway
OE Gaillimh

Date_____ Board No_____
Shire_____ RH_____

Patient label

Saolta

Research Questionnaire – Thyroid Biobank

1. Name? _____ Phone Number? _____
2. Date of birth? _____ Address? _____
3. What race/country are you from originally? E.g. (Irish Caucasian)_____
4. Gender? M / F

5. Have you ever had **thyroid cancer**? Yes☐ No☐
   a. If yes, what year? _____
   b. Could you feel a lump in your neck? _____
   c. What treatment did you have (chemo, radiotherapy?)_____
   d. What operation(s), if any, did you have? _____
   _____
   e. Did you have radioactive iodine treatment? _____
   f. Where/with who did you have your treatment?_____

6. Have you ever had any other type of **thyroid problem**? Yes☐ No☐
   a. What type? _____
   b. When? _____
   c. What treatment did you have? (Surgery, tablets?)_____
   d. Did you have radioactive iodine treatment? _____

7. Have you ever had any **other type of cancer**? Yes☐ No☐
   a. What type of cancer? / Where did it start? _____
   b. When? _____
   c. What treatment did you have? (Surgery, chemo, radiotherapy?)_____
   _____

Thank you so much for your participation. **NEXT PAGE →**

NUI Galway
OÉ Gaillimh

Date_____ Board No_____
Shire_____ RH_____

Patient label

Saolta

8. Do you have a **family history of thyroid cancer** (parent, sibling, child, aunt/uncle, niece/nephew, cousin, grandparent)? Yes☐ No☐

What relative?	Age of diagnosis (approx)?

9. Do you have a **family history of thyroid disease**, other than thyroid cancer? (parent, sibling, child, aunt/uncle, niece/nephew, cousin, grandparent)? Yes☐ No☐

What relative?	Thyroid Problem?	Age of diagnosis (approx)?

10. Do you have a **family history of other cancer** (parent, sibling, child, aunt/uncle, niece/nephew, cousin, grandparent)? Yes☐ No☐

What relative?	Cancer Type?	Age of diagnosis (approx)?

11. Is Multiple Endocrine Neoplasia (MEN) Syndrome in your family? Yes☐ No☐

Thank you so much for your participation.

NUI Galway
OÉ Gaillimh

Date_____ Board No_____

Shire_____ RH_____

Patient label

Saolta

For Medical Use Only:

Collection Site (eg Thy Ca clinic, wards, surgical clinic):_____

Operating consultant (eg Kerin, Quill, Young):_____

Sample type?_____Blood / Buccal swab_____Year of diagnosis: _____

Procedure 1 (eg FNA/lobectomy): _____

Procedure 2: _____

Procedure 3: _____

Procedure 4: _____

FNA 1 Histo & Bethesda:_____

FNA 2 Histo & Bethesda:_____

Cancer histology type (eg papillary, hurthle cell carcinoma, benign adenoma, Graves' etc):

_____

Size of each focus:_____ Total Size: _____ Largest focus: _____

No of Nodes Excised: _____ No of Nodes +ve: _____

Lympho-vascular invasion? _____ Extracapsular/thyroidal extension? _____

Local recurrence? _____ Year? _____ Distal recurrence? _____ Year? _____

MNG hx? _____

Has patient had RAI? _____

Notes_____

_____

_____

Thank you so much for your participation.

7.4 Questionnaire for participating controls

1. What is your name? _____ Contact No: _____

2. DoB? _____ Address: _____

3. What country are you from originally? _____

4. Have you ever had any type of thyroid disease? Yes☐ No☐
   a. What type? _____
   b. When? _____
   c. What treatment did you have? _____
   d. Did you have radioactive iodine treatment? _____

5. Have you had any type of cancer? Yes☐ No☐
   a. What type of cancer? _____
   b. When? _____
   c. What treatment did you have? _____

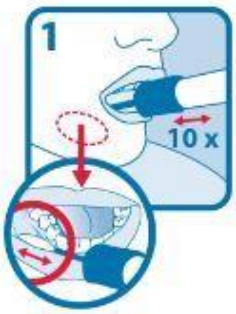
6. Do you have a family history of cancer (parents, siblings, aunts, uncles, cousins or grandparents)? Yes☐ No☐
   If yes, please list which relatives, their ages at diagnosis and type of cancer:
   _____
   _____
   _____
   _____

7. Do you have a family history of thyroid disease, other than thyroid cancer? (parents, siblings, aunts, uncles, cousins or grandparents)? Yes☐ No☐
   If yes, please list which relatives, their age at diagnosis and type of thyroid problem:
   _____
   _____
   _____
   _____

Thank you so much for your participation.

7.5 DNA Genotek ORAcollect•DNA | OCR-100 Collection Method

1



Open package and remove collector without touching sponge tip. Place sponge as far back in the mouth as comfortable and rub along the lower gums (see close up image) in a back and forth motion. Gently rub the gums 10 times. If possible, avoid rubbing the teeth.

2

Gently repeat rubbing motion on the opposite side of the mouth along the lower gums for an additional 10 times.

3

Hold the tube upright to prevent the liquid inside the tube from spilling. Unscrew the blue cap from the collection tube without touching the sponge.

4

Turn the cap upside down, insert the sponge into the tube and close cap tightly.

5

Invert the capped tube and shake vigorously 15 times.

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9 Chapter 9 – Manuscripts

Endocrine CONNECTIONS

Investigating the association of rs2910164 with cancer predisposition in an Irish cohort

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Abstract

Introduction: MicroRNAs (miRNAs) are small noncoding RNA molecules that exert post-transcriptional effects on gene expression by binding with cis-regulatory regions in target messenger RNA (mRNA). Polymorphisms in genes encoding miRNAs or in miRNA–mRNA binding sites confer deleterious epigenetic effects on cancer risk. miR-146a has a role in inflammation and may have a role as a tumour suppressor. The polymorphism rs2910164 in the *MIR146A* gene encoding pre-miR-146a has been implicated in several inflammatory pathologies, including cancers of the breast and thyroid, although evidence for the associations has been conflicting in different populations. We aimed to further investigate the association of this variant with these two cancers in an Irish cohort.

Methods: The study group comprised patients with breast cancer (BC), patients with differentiated thyroid cancer (DTC) and unaffected controls. Germline DNA was extracted from blood or from saliva collected using the DNA Genotek Oragene 575 collection kit, using crystallisation precipitation, and genotyped using TaqMan-based PCR. Data were analysed using SPSS, v22.

Results: The total study group included 1516 participants. This comprised 1386 Irish participants; 724 unaffected individuals (controls), 523 patients with breast cancer (BC), 136 patients with differentiated thyroid cancer (DTC) and three patients with dual primary breast and thyroid cancer. An additional cohort of 130 patients with DTC from the South of France was also genotyped for the variant. The variant was detected with a minor allele frequency (MAF) of 0.19 in controls, 0.22 in BC and 0.27 and 0.26 in DTC cases from Ireland and France, respectively. The variant was not significantly associated with BC (per allele odds ratio = 1.20 (0.98–1.46), $P = 0.07$), but was associated with DTC in Irish patients (per allele OR = 1.59 (1.18–2.14), $P = 0.002$).

Conclusion: The rs2910164 variant in *MIR146A* is significantly associated with DTC, but is not significantly associated with BC in this cohort.

Key Words
- thyroid
- endocrine cancers

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Background

The association between breast and thyroid disorders has been widely explored with a large amount of epidemiological evidence linking breast and thyroid malignancies. However, the extent and explanation for this association have remained ill-defined. A recent meta-analysis has revisited the possibility of such an association and has confirmed the existence of, and quantified the increased co-occurrence of breast and differentiated thyroid cancer (DTC) (1). Both cancers occur predominantly in females, and there is a significantly increased risk of developing thyroid cancer as a second primary malignancy following a diagnosis of breast cancer (BC) and vice versa (2, 3, 4, 5). Mutations in *PTEN* have long been known to predispose to both types of malignancy as part of the *PTEN* hamartoma tumour syndrome (6). A relationship between BC and benign thyroid disease has also been hypothesised (7), given the common iodine transport mechanism (8, 9), prevalence of anti-thyroid peroxidase (TPO) autoantibodies in BC patients (10, 11, 12) and the role of thyroid hormone receptor B in BC (13). A non-syndromic monogenic disorder predisposing to breast and thyroid cancers has been postulated but has not, as yet, been identified (14). However, it is possible that this association may be explained by overlapping moderate- or low-penetrance breast-thyroid cancer genetic susceptibility loci (15).

MicroRNAs (miRNAs) are crucial elements in the regulation of gene expression and are involved in a host of physiological and pathological processes. A substantial proportion of the human transcriptome is subject to regulation by miRNAs (16). MicroRNA genes are transcribed from endogenous DNA into primary miRNA transcripts (pri-miRNA), which are then processed by Drosha-containing complexes to form hairpin structures called pre-miRNAs. Pre-miRNAs are then transported into the cytoplasm and processed further by a Dicer-containing complex, which acts to excise the hairpin loop. Binding of miRNA to target messenger RNA (mRNA) leads to translational suppression or mRNA degradation (17). Partial complementarity is often sufficient for binding (16), meaning that individual miRNAs may have hundreds of different mRNA targets, and the individual mRNA target can be regulated by many different miRNAs leading to a rich and complex miRNA–mRNA network. The potential complexity of the miRNA–mRNA network can be exemplified by the miR-146 family of microRNAs. This family includes two closely related but genetically distinct microRNAs, miR-146a and miR-146b, differing only at two nucleotides in the 3′ region of the mature sequences (17).

These miRNAs are critical in a number of immune and inflammatory response pathways and are activated differentially by NF-KB and in response to pro-inflammatory cytokines (17). miR-146a has a number of molecular targets involved in innate and adaptive immune responses; cell proliferation, invasion and metastasis; including, among others, TRAF6 (17), IRAK1 (17), IRAK2 (18), EGF-R (19), NOTCH1 (20) and ROCK1 (21). miR-146a upregulation is mediated by BRAF and NRAS oncoproteins (20).

The typical human genome varies from the reference sequence at 4.1–8 million sites (22), and the majority of this variation is attributable to small indels and single nucleotide polymorphisms (SNPs). The vast majority of these SNPs are benign, but they may become relevant functionally and clinically if they occur in a critical binding site or regulatory region (23). A single nucleotide variant (rs2910164: G>C) in the precursor stem region of *pre-miR-146a* is thought to reduce the stability of the pri-miR and affect processing of pri- to pre-miRNA, thus impacting expression of mature miR-146a (24). This variant has been implicated in a host of malignant and non-malignant inflammatory conditions such as hepatocellular (25) and gastric cancers (26), coronary artery disease (27), inflammatory bowel disease (28) and multiple sclerosis (29, 30). Some authors report an association between the variant in heterozygous (GC), but not homozygous (CC) states and an increased risk of papillary thyroid cancer compared to wild-type genotype (GG) (24), while others report an association with both heterozygous and homozygous states (31). Data with respect to this association are conflicting, with other groups failing to show an association with hetero- or homozygous genotypes (32, 33). Similarly, there are conflicting reports of the impact of the variant allele on mature miR-146a expression, with some authors reporting reduced expression (24), and others overexpression (34).

A possible association between the rs2910164 variant and BC was suggested after a report by Shen and coworkers suggested an impact of age of onset of familial BC on Chinese patients (34). An Italian study of a small cohort of carriers with *BRCA1/BRCA2* mutations also suggested an influence of age of onset of disease, but not on risk overall (35). However, no association between age at diagnosis or disease risk and genotype was identified in a larger study of *BRCA1/BRCA2* mutation carriers from Europe and USA

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(36) or in a different Chinese cohort of sporadic cases (37). It has been postulated that ethnicity may significantly modify the association between miRNA polymorphisms and cancer risk (38). An association between genotype and sporadic BC risk and phenotype has been investigated in variably powered studies from China (37), Italy (35, 39), Germany (39), Spain (40), Australia (41), Saudi Arabia (42), India (43) and Iran (44, 45, 46); and in numerous meta-analyses with conflicting results (26, 47, 48, 49). To date, no Irish samples have been included in such analyses.

The aims of this study were to investigate the association between the variant allele of rs2910164 in *MIR146A* and predisposition to breast and differentiated thyroid cancers in an Irish patient sample and to investigate the frequency of the variant in a distinct patient population from the South of France.

Methods

Study samples

Case–control analyses were undertaken, comparing genotypic and allelic frequencies of the variant in patients with BC and in those with DTC, to frequencies in unaffected controls.

Unselected patients with confirmed *in situ* or invasive BC ($n=534$) were recruited via a symptomatic and screening BC tertiary referral centre (Galway University Hospital). Of these, 7 were found to carry a pathogenic mutation in *BRCA1* or *BRCA2* and were excluded from analysis. Two other individuals were found to carry pathogenic mutations in another BC susceptibility gene (*CHEK2*, *CDH1*) and were also excluded.

Patients were recruited from thyroid cancer treatment clinics at tertiary centres in the West of Ireland and South of France as part of a collaborative multicentre study to establish a thyroid cancer biobank at the Discipline of Surgery in the Lambe Institute for Translational Research, based in Galway University Hospital. Patients with a histological confirmation of DTC were included ($n=269$). Exclusion criteria included benign thyroid disease, medullary thyroid cancer, or known pathogenic germline mutations in cancer predisposition genes.

Individuals were included as controls if they did not have a current or previous diagnosis of cancer (not including non-melanomatous skin cancer), if they did not have a first-degree familial history of breast, ovarian or thyroid cancers and if they were aged over sixty years. Controls were recruited from non-oncological outpatient clinics, and from volunteers in the community.

Written and informed consent was obtained from each patient, and the study was approved by the Institutional Ethics Review Board at Galway University Hospital.

Data pertaining to tumour clinico-pathological characteristics and previous germline genetic testing were recorded from hospital histopathology and genetic testing reports. Information regarding personal and familial medical history was self-reported by patients.

DNA extraction

Participants recruited in hospital were asked to provide a 10 mL whole blood sample, and those recruited from the community were asked to provide a salivary sample collected using the DNA Genotek Oragene 575 collection kit. DNA was extracted manually by ethanol precipitation from whole blood or saliva, and qualified and quantified using nanodrop spectrophotometry. DNA was deemed to be of suitable purity if the ratio of the absorbance at 260 and 280 nm measured approximately 1.8 (50).

Genotyping

Genotyping was performed by PCR using a TaqMan assay (Applied Biosystems) containing allele-specific probes and a PCR primer pair to detect the specific variant under investigation. Each allelic discrimination reaction mix contained 1 µL TaqMan SNP genotyping assay, 10 µL TaqMan SNP genotyping Master Mix and 40 ng/9 µL genomic DNA. RT-PCR was performed using ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 95°C for 10 min to facilitate activation of DNA polymerase, and 40 cycles of amplification, with denaturation at 95°C for 15 s followed thereafter by annealing and extension at 60°C for one minute. The assay for rs2910164 SNP was manufactured such that reporter dyes were tagged to the 5′ end of alternative allele probes (VIC to the variant C allele probe, FAM to the G allele probe (Thermo Fisher Scientific; www.thermofisher.com/order/genome-database/browse/genotyping/keyword/rs2910164)) and a non-fluorescent quencher at the 3′ end of the respective probes). VIC dye fluorescence only was interpreted as homozygosity for the C allele, FAM dye fluorescence only as homozygosity for the G allele and fluorescence signals from both dyes as heterozygosity. Allelic discrimination plots were generated, with automatically interpreted FAM and VIC fluorescent signals plotted on *X* and *Y* axes. Automated

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genotype calling was confirmed manually by interrogation of multicomponent plots.

Statistical analysis

Data were analysed using SPSS, version 24. Continuous data were assessed for normality using the Kolmogorov–Smirnov tests and analysed using parametric or non-parametric tests as appropriate. Normal data were expressed as mean ± standard deviation, and non-normally distributed data as median (range). Categorical data were assessed using chi-squared tests. The frequency of the variant was assessed for Hardy–Weinberg equilibrium using chi-squared test. Case–control analyses were performed between patients of matched ethnicity.

Results

Considering the Irish cohort primarily, samples from 724 controls, 523 patients with BC, 136 patients with DTC and 3 patients with dual-primary breast and thyroid cancer were successfully genotyped for rs2910164. The clinical and pathological characteristics of the patient cohort are outlined in Tables 1 and 2. The variant was proven to be in Hardy–Weinberg equilibrium in both case cohorts and in controls.

The frequency of the minor allele was higher in BC cases (0.22) than controls (0.19), but the per allele odds ratio for the C allele did not achieve statistical significance (OR 1.20 (0.98–1.46), $P=0.07$). Neither hetero- nor homozygous genotypes were associated with BC in this patient population (Tables 3 and 4). No significant association was detected between genotype and age at diagnosis of BC ($P=0.197$, Kruskal–Wallis test) or molecular subtype of BC ($P=0.715$, X^2) (Table 5). No association was evident between genotype and T-stage ($P=0.689$, X^2), absolute tumour size ($P=0.327$, Kruskal–Wallis test) or nodal status ($P=0.861$, X^2).

The frequency of the minor allele was significantly higher in DTC cases (0.27) than controls (0.19). The C allele variant was significantly associated with DTC in both heterozygous (OR 1.66 (1.13–2.44), $P=0.009$) and homozygous genotypes (OR 2.24 (1.05–4.78), $P=0.03$) (Tables 3 and 4). When association was analysed by gender, the association remained significant for females. The association also retained significance when histological subtype was considered. When papillary subtypes of thyroid cancer only ($n=110$) were considered, the risk conferred by the CG genotype was 1.55 (1.01–2.38), $P=0.04$; and by the CC genotype, 2.81 (1.3–6.05), $P=0.006$ (Table 5).

We did not identify an association between genotype and age at diagnosis of DTC ($P=0.47$, ANOVA) (Fig. 1). There was no appreciable association between genotype and nodal status ($P=0.728$, X^2) or T-stage ($P=0.079$, X^2).

All three female patients diagnosed with both breast and thyroid primary malignancies were found to be of CG genotype.

One hundred and thirty patients with DTC were recruited from a tertiary centre in the South of France and genotyped successfully for this variant (Tables 6 and 7). This sample demonstrated much greater diversity in terms of ethnic origin – with the majority identifying as 'French Caucasian' ($n=90$, 69%), but significant patients reporting other European ($n=20$, 15%), Asian ($n=7$, 5%) or North African origin ($n=10$, 8%). The frequency of the variant allele in this population was 0.26. There was

Table 1 Irish patient characteristics.

	Breast cancer (*N* (%))	**Thyroid cancer** (*N* (%))	**Controls** (*N* (%))
Total	526*	139* (100)	724 (100)
Gender			
Male	3 (1)	27 (19)	53 (7)
Female	523* (99)	112* (81)	671 (93)
Age at diagnosis (cases) or sampling (controls)			
Median (range)	53 (30–88)	42 (16–84)	70 (60–93)
Mean ± S.D.	55.14 ± 11.11	45.33 ± 15.01	70.72 ± 6.71
Age groups			
15–39	28 (5)	48 (35)	0
40–49	149 (28)	29 (21)	0
50–64	229 (44)	32 (23)	124 (17)
≥65	104 (20)	13 (9)	600 (83)
Unknown	16 (3)	17 (12)	0 (0)

*Including three female patients with breast and thyroid cancer.

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Table 2 Tumour clinico-pathological features.

Breast cancer (*N*=526)		**Thyroid cancer** (*N*=139)	
Histology			
Ductal	397 (75)	Papillary	112 (81)
Lobular	78 (15)	Follicular	27 (19)
Colloid	12 (2)		
Other	19 (4)		
Missing	20 (4)		
Molecular subtype			
Luminal A	344 (65)		
Luminal B	61 (12)		
Her2-overexpressing	28 (5)		
Triple negative	53 (10)		
Unknown	40 (8)		
T-stage			
Is	33 (6)		
1	182 (35)	1	57 (41)
2	216 (41)	2	45 (32)
3	39 (7)	3	25 (18)
4	16 (3)	Unknown	12 (9)
Unknown	37 (7)		
N-stage			
0	250 (48)	0	38
1	130 (25)	1	21
2	62 (12)		
3	27 (5)		
Missing	57 (11)	Not assessed	80

Table 3 Genotypic and allelic frequencies in Irish patients.

Genotype	**Control** (*N*=724)	**DTC** (*N*=139)	**Breast** (*N*=526)
GG	480	74	326
CG	215	55	171
CC	29	10	29
C allele	273	75	229
G allele	1175	203	823
Minor allele frequency	0.19	0.27	0.22
Male individuals only			
Male	**Control** (*n*=53)	**DTC** (*n*=27)	**Breast** (*n*=3)
GG	35 (66)	13 (48)	2 (67)
CG	16 (30)	12 (44)	0
CC	2 (4)	2 (7)	1 (33)
C allele	20	16	2
G allele	86	38	4
Minor allele frequency	0.19	0.30	0.33
Female individuals only			
Female	**Control** (*n*=671)	**DTC** (*n*=112)	**Breast** (*n*=523)
GG	445	61	324
CG	199	43	171
CC	27	8	28
C allele	253	59	227
G allele	1089	165	819
Minor allele frequency	0.19	0.26	0.22

Table 4 Genotypic and allelic odds ratio (Irish patients).

	Odds ratio (95% CI)		
	Thyroid cancer		
	Per C allele	CG vs GG	CC vs GG
Overall	1.59 (1.18–2.14) *P*=0.002	1.66 (1.13–2.44) *P*=0.009	2.24 (1.05–4.78) *P*=0.03
Male	1.81 (0.85–3.87) *P*=0.13	2.02 (0.76–5.39) *P*=0.16	2.69 (0.34–21.14) *P*=0.33
Female	1.54 (1.11–2.14) *P*=0.01	1.58 (1.03–2.41) *P*=0.03	2.16 (0.94–4.97) 0.06
	Breast cancer		
	Per C allele	CG vs GG	CC vs GG
Overall	1.20 (0.98–1.46) *P*=0.07	1.17 (0.92–1.5), *P*=0.21	1.47 (0.86–2.51) *P*=0.15
Male	2.15 (0.37–12.57) *P*=0.40	0 (n/a)	8.75 (0.54–142.69) *P*=0.77
Female	1.19 (0.98–1.46) *P*=0.09	1.18 (0.92–1.51) *P*=1.19	1.42 (0.82–2.46) *P*=0.20

no significant difference in age at diagnosis (P=0.984), T-stage (0.066) or nodal involvement (P=0.945) between genotypes (Table 8).

Discussion

The role of miRNA-146a and miRNA-146b in inflammation, immune function and epithelial cell homeostasis and their reported roles in inhibition of invasion and metastasis, make them seductive candidates as cancer susceptibility genes. The function of miR-146a, as is true for other microRNAs, appears to be tissue, as well as context specific (51). Different studies have variably categorised miR-146a as tumour suppressor or oncogenic microRNA depending on the tissue of interest (52, 53).

The expression of these miRNAs has been shown to be upregulated in basal-like BC cell lines, a subtype commonly associated with BRCA1 deficiency, compared to luminal subtypes. Binding of these miRNAs to target sites in the 3′UTR of BRCA1 can also downregulate its expression, leading to increased cellular proliferation (54). However, miRNA-146b has also been shown to be upregulated in healthy basal mammary epithelial cells (55). It has also been reported that upregulation of miR-146a/b by BRMS1 leads to inhibition of invasion and metastasis of MDA-MB-231 human breast carcinoma cells (56), by subsequent downregulation of NF-kB through the targets IRAK1 and TRAF6 (57). In other studies, such upregulation was associated with an anti-apoptotic effect in p53-deficient breast tumours (52). The expression of genes involved in the NF-KB pathway and

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Table 5 Genotypic odds ratios depending on molecular subtype of breast cancer or histological subtype of thyroid cancer.

				Genotypic odds ratio (95% CI)			
	GG	**GC**	**CC**	**GC vs CC**		**GG vs CC**	
Molecular subtypes of breast cancer							
Luminal A ($n=342$)	219	106	17	2.02 (1.76–5.39)	$P=0.16$	2.69 (0.34–21.14)	$P=0.33$
Luminal B ($n=61$)	35	22	4	1.4 (0.8–2.45)	$P=0.23$	1.89 (0.63–5.68)	$P=0.25$
Her2-overexpressing ($n=28$)	18	7	3	0.87 (0.36–2.11)	$P=0.75$	1.07 (0.24–2.68)	$P=0.92$
Triple negative ($n=52$)	31	20	2	1.44 (0.8–2.58)	$P=0.22$	2.76 (0.77–9.91)	$P=0.11$
Histological subtypes of thyroid cancer							
Papillary ($n=110$)	59	41	10	1.55 (1.01–2.38)	$P=0.04$	2.81 (1.3–6.05)	$P=0.006$
Follicular ($n=26$)	15	11	0	1.64 (0.74–3.62)	$P=0.2$	n/a	

regulation of apoptosis may also be dependent on pre-miR-146a genotype (53).

Considering rs2910164 in particular, *in vitro* studies have suggested that mature miRNA-146a levels are increased in MCF7 cell lines transfected with pcDNA3.3-miR-146C vs pcDNA3.3-miR-146G, which the authors postulate to be related to increased binding capacity of miR-146a to BRCA1 in the presence of the C-variant (33). However, we, and other authors, have previously shown that circulating miR-146a levels are reduced in the presence of the C allele in patients with BC (58, 59).

miR-146a and miR-146b have been shown to be upregulated and associated with adverse prognostic features, progression and invasion in papillary (60), follicular (61) and anaplastic (62) thyroid cancers. This effect may be mediated through associations with NF-KB (62), ST8SIA4 (61) or RARB (63, 64). A previous study by Jazdzewski and coworkers postulated that different genotypes did not show an association between the homozygous rs2910164 genotype and disease, which they attribute to differential production of mature miRNA in tumours, with heterozygotes producing three alternate isoforms of the miRNA (miR-146a from leading strand, and miR-146a*G and miR-146a*C from passenger strand), with different sets of target genes (24, 65).

Our data support a possible association between the variant allele of rs2910164 and DTC in this patient population. Furthermore, an allele dosage effect was observed, with homozygous genotypes associated with increased odds of disease compared to heterozygotes. In our study, 7% of patients were homozygous for the variant compared to 4% of controls, while in the cohort of the study by Jazdzewski and coworkers the homozygote genotypic frequency was 2.9% in cases compared to 6.6% in controls (24). This study included samples from three ethnically distinct populations (Finland, Poland and USA); with approximately equal representation of cases from each cohort, but over half of the controls were Polish. We have demonstrated that the frequency of the variant allele in French cases is 0.26, which is comparable to that reported in our Irish cases (0.27), and to the MAF in Finnish cases (0.27) (24) and comparatively higher than

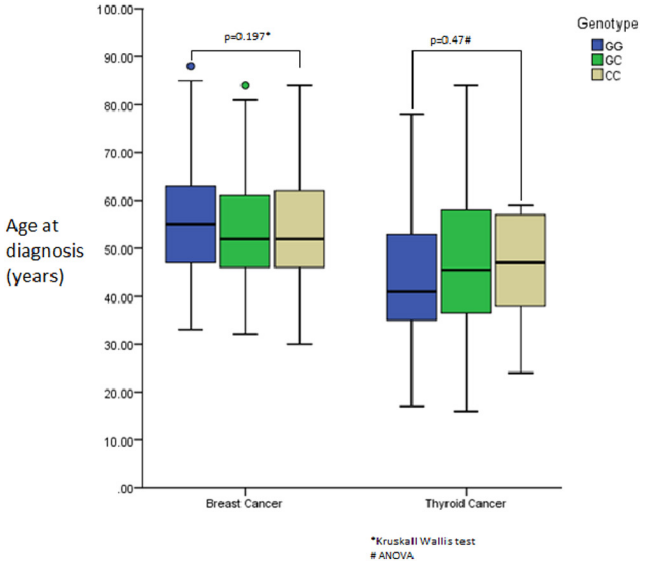

Figure 1
Lack of association between genotype and age at diagnosis.

Table 6 Characteristics of French patients.

Patient characteristic	***N*** (%)
Ethnicity	
French Caucasian	90 (69)
Other European Caucasian	20 (15)
Asian	7 (5)
African	10 (8)
Other	3 (2)
Minor allele frequency	0.26
Mean age at diagnosis ± S.D. (years)	47.71 ± 15.28
Gender	
Male	37 (29)
Female	93 (72)
Histopathological subtype	
Papillary	119 (92)
Follicular	11 (9)

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Table 7 Genotypic frequency in French cohort.

	Patient genotype			
	GG	GC	CC	Significance
N (%)	72 (55)	49 (38)	9 (7)	
Minor allele frequency	0.26			
Mean age at diagnosis ± S.D. (years)	47.85 ± 14.56	47.65 ± 16.27	46.89 ± 17.14	*P* = 0.984, ANOVA
T-stage				
1	40	21	3	0.066, X^2
2	13	12	6	
3	17	12	0	
4	1	0	0	
Unknown	1	4	0	
Nodal status				
0	17	9	2	0.945, X^2
1	22	10	2	
Not assessed	32	27	5	
Unknown	1	3	0	

the MAF reported in the Polish (0.22) (24) or UK (0.24) (32) cohorts, but lower than that reported in American cases (0.30) (24) and considerably lower than that reported in Chinese patients (0.57). A cohort of French controls is required for comparative analyses before any conclusion can be made regarding the significance of this variant in the French population, as there is obvious variability in frequency of the variant that can be demonstrated across different populations. This high MAF in DTC cases merits further investigation in a larger study with controls of matched ethnicity. Variability in frequency of the variant across different populations is exemplified by the different MAF demonstrated in our cohort compared to our nearest geographical neighbours in the United Kingdom, where the MAF in both control and case cohorts was 0.24. The UK study therefore did not support an association

Table 8 Lack of association of genotype with age, T-stage, nodal status.

	Genotype			
	GG	GC	CC	Significance
Breast cancer				
Age	55 (33–88)	52 (32–84)	52 (30–84)	0.197 (Kruskall–Wallis)
T-stage				
Is	24	8	1	0.689, X^2
1	105	65	12	
2	133	72	11	
3	26	10	3	
4	12	3	1	
Unknown	26	10	1	
Tumour size	25 (2–100)	23 (2–116)	25 (2–110)	0.327 (Kruskall–Wallis)
Nodal status				
0	158	77	15	0.861, X^2
1	80	42	8	
2	36	22	4	
3	19	8	0	
Thyroid cancer				
Age	43.89 ± 14.69	47.45 ± 16	46 ± 12.44	0.470 (ANOVA)
T-stage				
1	38	15	4	0.079, X^2
2	21	22	2	
3	10	12	3	
Unknown	5	3	1	
Nodal status				
0	24	12	2	0.728, X^2
1	11	7	2	
Not assessed	39	33	6	

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of the variant with disease. Our criteria for inclusion of participants as controls was much more stringent than in this study, where controls were recruited from the national blood donor service, the 1958 Birth cohort, and from a separate colorectal cancer susceptibility gene discovery project (COloRectal Gene Identification study (CORGI)). Furthermore, although the number of cases included in this study was greater (cases: 748 vs 139; controls: 2857 vs 724); the population in the United Kingdom is 65.6 million (66), almost 14 times greater than that in the Republic of Ireland (approximately 4.7 million (67)). Our sample therefore represents a greater proportion of the national population.

We applied rigorous selection criteria to controls, specifying that they must be aged at least 60 years; the rationale being that patients with a genetic predisposition to cancer are more likely to be affected at younger ages, and older individuals have the lowest residual lifetime possibility of developing cancer. Indeed, the median age at diagnosis of thyroid cancer in our cohort was 45 years (range 16–84). The controls in other studies were young or age-matched unaffected individuals recruited from outpatient clinics or as part of another study (24, 68, 69). In an Italian study, no association between the variant and thyroid cancer was described, but the authors do not describe the ages of the control individuals (43). Young patients have a higher lifetime probability of developing a cancer that may be related to an underlying predisposition.

Previous studies have suggested that gender may influence the strength of the association of rs2910164 with disease (70); potentially because of the regulation of miRNA-146a expression by oestrogen in immune cells (71). In this study, the strength of the association between the variant and thyroid cancer retained significance in female patients, but did not in male patients. A limitation of this study however is the small number of male controls, and this subgroup is underpowered to draw any formal conclusion from the analysis.

As the variant in question is a transversion involving two nucleotides of a Watson–Crick pair; it is important that the orientation of the DNA strand on which the variant is called and the method by which genotyping is performed are considered. Previous meta-analyses have described the multitude of methods by which genotyping of this variant has been performed in different cohorts (72). It is important not just to consider the method, but also the orientation of the primers in the assays utilised and indeed the assembly build on which their design is based. A lack of clarity about this point may have contributed to the apparently discordant results in the literature to date; a confounding factor that has been reported in the investigation of other SNPs (73). The situation in this context is further complicated by the differing frequencies and indeed impact of the variant allele on different populations, especially between East Asian and European Caucasian populations (69, 72; http://exac.broadinstitute.org/variant/5-159912418-C-G).

The technology at our disposal to investigate and identify genetic variation has improved dramatically in recent years. It is therefore likely that the 'low-hanging fruit' – the highly penetrant monogenic cancer predisposition syndromes – have already been identified. The missing heritability of cancer is likely to be attributable to low-penetrance alleles in multiple genes. While individually these polymorphisms may confer small effect sizes, the cumulative risk conferred by inheritance of multiple low-penetrance alleles may possibly approach that of the high-risk monogenic disorders. As we start to develop algorithms to include data from low-penetrance alleles into BC risk estimation (74), we must endeavour to do the same for less common malignancies – particularly those of which the genetic architecture is, as yet, poorly defined.

This study highlights a number of key points. In this cohort, the variant rs2910164 appears to be associated with DTC, but does not have a clear association with BC risk, nor age of disease onset or molecular subtype of BC. The clinical utility of the identification of this variant in a patient sample is, as yet, undetermined, given the numerous potential inflammatory benign and malignant disease processes in which miR-146a has a role; and the differential frequency and influence of this variant across populations. This study is limited by a relatively small number of samples from patients with DTC. However, we believe this sample to be representative of Irish patients with the disease, considering that we have strongly matched for ethnicity in a population of only 4,757,976 (67, 75) with a thyroid cancer incidence of 3.61/100,000 (75).

The possibility of variant allele misalignment between different studies does exist, and a robust meta-analysis, accounting for this, may further elucidate the association between the variant allele at this locus and cancer predisposition.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Differentiated Thyroid Cancer: How Do Current Practice Guidelines Affect Management?

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Keywords
Differentiated thyroid cancer · Papillary cancer · Follicular cancer · Guidelines · Risk stratification · British Thyroid Association

Abstract
Background: International best-practice guidelines recommend completion thyroidectomy and radioiodine remnant ablation (RRA) for patients with differentiated thyroid cancer (DTC) >4 cm or with specific risk factors. Patients with DTC <1 cm without risk factors are recommended for lobectomy alone. Indications for aggressive surgery and RRA are less clearly defined for tumours measuring 1–4 cm. A personalised approach to decision-making is recommended. ***Objectives:*** This study assesses therapeutic approaches to DTC as compared to the current British Thyroid Association (BTA) clinical practice guidelines. We ascertained the effect of equivocal guidance in the 1–4 cm tumour cohort on contemporary practice patterns. ***Methods:*** Data were obtained from a prospectively maintained thyroid cancer database of patients treated for DTC in a tertiary referral centre at the University Hospital Galway. Consecutive patients attending a dedicated thyroid cancer clinic between August 2014 and August 2017 were included. Clinicopathological characteristics and management strategies were assessed. ***Results:*** Ninety-four percent (n = 168/178) of patients were surgically managed in adherence with guidelines. A minority (n = 10) received surgery not aligned with guidelines. Ninety-seven percent (n = 172/178) of RRA treatment decisions were in accordance with guidelines. The BTA guidelines recommended a personalised decision-making approach for 18.0% (n = 32) and 44.9% (n = 80) of surgery and RRA treatment decisions, respectively. The more aggressive, treatment-driven approach was typically favoured by the multidisciplinary team, with 97% (n = 31/32) undergoing completion thyroidectomy and 100% (n = 80) proceeding to RRA. ***Conclusions:*** Management of DTC at our institution closely adheres to contemporary clinical practice guidelines. The finding of more aggressive management in those requiring a personalised decision-making approach highlights the requirement for improved risk stratification in this cohort to rationalise management strategies.

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Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, accounting for 1% of all cancers, with an incidence of 162 cases per year in Ireland [1]. The National Cancer Registry Ireland reports increasing incidence of TC from 60 per year during the mid 1990s to 160 per year during the late 2000s [1]. Similar trends are reported worldwide, including the United Kingdom, the United States, and South Korea [2–4]. This observed increased incidence primarily comprises differentiated TC (DTC), without a concomitant increase in mortality, suggesting potential overdiagnosis of indolent pathology [4–7]. In Ireland, the 5-year TC survival rate has improved from 71% (1994–1998) to 91.8% (2010–2014), while the 5-year DTC-specific survival is 98% [1]. Data from 51,061 DTC patients from the US Surveillance, Epidemiology, and End Results database report an overall 5-year survival of 96.5% [8]. The mainstay of TC treatment involves surgical resection with or without adjuvant radioiodine remnant ablation (RRA); it follows that overdiagnosis of DTC results in potentially avoidable morbidity arising from surgical or RRA therapies.

The key recommendations of the British Thyroid Association (BTA) 2014 guidelines for the management of thyroid cancer include diagnostic lobectomy for those with Thy3 or Thy4 fine-needle aspiration cytology (FNAC). Total thyroidectomy (TT) is advised for patients with Thy5 FNAC or with confirmed DTC following diagnostic lobectomy where tumour size exceeds 4 cm or measures any size with risk factors including multifocal, bilateral, extrathyroidal, or familial disease, and those with clinically or radiologically involved nodes or distant metastases. Lobectomy alone is deemed sufficient for patients with unifocal papillary thyroid microcarcinoma (microPTC, <1 cm) without risk factors; these include multifocality, larger size (6–10 mm), extrathyroidal extension, poor differentiation, and desmoplastic fibrosis or an infiltrative growth pattern. There is a paucity of peer-reviewed randomised or prospective studies to support an advantage of TT over lobectomy in patients with unifocal tumours measuring 1–4 cm without risk factors. In these cases, the BTA guidelines recommend a personalised decision-making approach, which advocates a shared doctor-patient decision-making process in conjunction with multidisciplinary team input, with due consideration for recurrence risk, patient comorbidities, and personal circumstances and values [9].

The BTA recommends RRA for all patients with DTC >4 cm or any size with gross extrathyroidal extension or distant metastases. Patients with tumours ≤1 cm without risk factors do not benefit from RRA. Personalised decision-making is advised for those with tumours measuring 1–4 cm without risk factors [9].

We aimed to describe the patterns of DTC presentation and treatment strategies and to assess the degree of adherence to clinical practice guidelines (BTA 2014) in patients treated for DTC at our institution. We further examined those patients where a personalised decision-making approach was recommended and how this subset was managed in the context of equivocal guidelines.

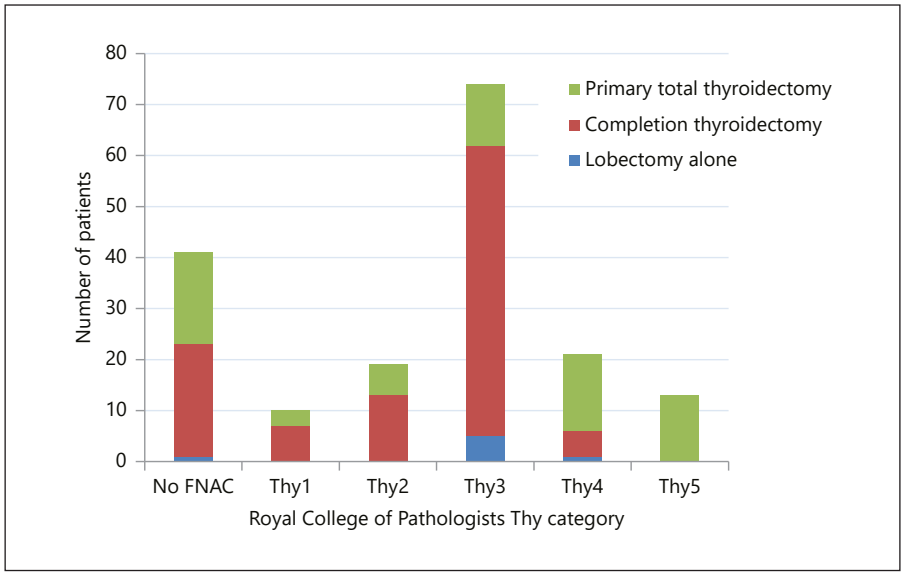
Materials and Methods

Data were prospectively recorded from consecutive patients attending a specialist TC clinic in a tertiary referral centre (University Hospital Galway) over 3 years (August 2014–2017). Data collection was undertaken at the time of blood and sputum sample collection for inclusion in the University Hospital Galway TC BioBank. Patient demographics, tumour histology, and staging parameters were recorded, in addition to surgical and RRA management strategies. All patients were discussed in an endocrine surgery multidisciplinary team. TC staging and FNAC were designated as per the AJCC Cancer Staging Manual (7th edition) and the UK Royal College of Pathologists thyroid cytology guidelines, respectively [10, 11]. The BTA guidelines for the management of thyroid cancer were accepted as best-practice guidelines [9]. Data recording and statistical analysis were performed using Microsoft Excel and IBM SPSS v22. Pearson's χ^2 test was used to compare categorical variables, while one-way ANOVA with Tukey post hoc analyses was utilised for comparing three or more categorical, independent samples.

Results

Data were collected from 178 consecutive patients. Of these, 130 patients (73%) were female. The median age at diagnosis was 43.5 years (range 15–83 years). One hundred and fifty-two patients (85%) had papillary TC while 26 (15%) had follicular TC. The median tumour size was 26 mm (range 1–110 mm). Multifocal disease was present in 36% of patients ($n = 64$). Lymphovascular invasion and extracapsular extension were evident in 33% ($n = 58$) and 31% ($n = 55$) of tumours, respectively. Forty-eight percent ($n = 86$) had lymph nodes excised, with a mean harvest of 7.6 ± 8.5 nodes. Thirty-four patients had confirmed lymph node metastases, representing 40% of those with nodes excised and 19% of all patients. The average number of positive nodes retrieved was 4.7 ± 4.4.

FNAC results were available for 77% of patients ($n = 118$) (Fig. 1). All 13 patients with Thy5 FNAC underwent



Color version available online

Fig. 1. Surgical management per Royal College of Pathologists Thy category.

Table 1. Clinicopathological parameters based on surgical outcome

	Primary TT	Lobectomy alone	Lobectomy followed by completion thyroidectomy
Number of patients	67 (38%)	6 (3%)	105 (60%)
Female	51 (76%)	2 (33%)	77 (73%)
Age, years	46±16	50±23	46±16
Preoperative FNAC Thy1	3 (5%)	0 (0%)	7 (7%)
Preoperative FNAC Thy2	6 (9%)	0 (0%)	13 (12%)
Preoperative FNAC Thy3	12 (18%)	5 (83%)	57 (54%)
Preoperative FNAC Thy4	15 (22%)	1 (17%)	5 (5%)
Preoperative FNAC Thy5	13 (19%)	0 (0%)	0 (0%)
No preoperative FNAC	18 (27%)	0 (0%)	23 (22%)
Papillary (final histology)	61 (91%)	5 (83%)	86 (82%)
Follicular (final histology)	6 (9%)	1 (17%)	19 (18%)
Size, mm	22 (2–110)	5.25 (2–65)	30 (1–110)
Multifocal	26 (39%)	0 (0%)	38 (36%)
Nodal disease	25 (37%)	0 (0%)	9 (9%)
Lymphovascular invasion	29 (43%)	1 (17%)	28 (27%)
Extracapsular extension	23 (34%)	1 (17%)	31 (30%)

Values are presented as *n* (%), mean ± SD, or median (range). FNAC, fine-needle aspiration cytology; TT, total thyroidectomy.

primary TT. Primary TT was also performed for 3, 6, 12, and 15 patients with Thy1–4 FNACs, respectively. Of these 36 patients, 24 had features identified on preoperative imaging indicating TT (size >4 cm, lymphadenopathy, extracapsular or bilateral disease). TT was performed for benign indications (e.g., multinodular goitre, Graves' disease) in 9 patients, while 3 patients had no definitive indication for upfront TT.

Surgery

Thirty-eight percent of patients (n = 67) underwent primary TT. Sixty-two percent (n = 111) had thyroid lobectomy, with 95% of those (n = 105/111) proceeding to completion thyroidectomy (Table 1). Six patients (3%) had lobectomy alone; these were more likely to be male (68%, n = 4/6) compared to those who proceeded to completion thyroidectomy (27%, n = 28/105) (p = 0.031). Tumour size >4 cm, multifocality, and extracapsular exten-

Table 2. Surgical management compliance with the BTA 2014 guidelines

	Patients	Compliant with BTA guidelines
All patients	178 (100%)	168 (94%)
<1 cm	27 (15%)	18 (67%)
1–4 cm	113 (63%)	113 (100%)
>4 cm	38 (21%)	37 (97%)
Multifocal	64 (36%)	64 (100%)
Lymphovascular invasion	58 (33%)	57 (98%)
Extracapsular extension	55 (31%)	54 (98%)
Age >45 years	86 (48%)	79 (92%)

Values are presented as *n* (%). BTA, British Thyroid Association.

Table 3. Clinicopathological details for patients where the BTA recommended a personalised decision-making approach (*n* = 178)

	PDM recommended for surgical management (*n* = 32)	PDM recommended for RRA management (*n* = 80)
Had thyroidectomy/RRA	31 (97%)	66 (83%)
Female	25 (78%)	66 (83%)
Age, years	37±10	43±15
Papillary cancer	28 (88%)	70 (88%)
Follicular cancer	4 (13%)	10 (12%)
Size, mm	21 (2–40)	22 (10–40)
<1 cm	8 (25%)	1 (1%)
1–4 cm	24 (75%)	79 (99%)
>4 cm	0 (0%)	0 (0%)
Multifocal disease	4 (all microPTC)	30 (38%)

Values are presented as *n* (%), mean ± SD, or median (range). BTA, British Thyroid Association; microPTC, papillary thyroid microcarcinoma; PDM, personalised decision-making; RRA, radioiodine remnant ablation.

sion were associated with an increased rate of completion thyroidectomy compared to lobectomy alone ($p = 0.044$, $p = 0.027$, and $p = 0.044$, respectively). No significant association was observed between histological subtype or age and surgical procedure.

Ninety-four percent of patients ($n = 168/178$) were surgically managed in strict adherence with the BTA guidelines (Table 2). Of those not aligned with the guidelines ($n = 10$), 1 had a tumour >4 cm at lobectomy, but declined completion thyroidectomy. Nine patients had tumours <1 cm without risk factors, of whom 4 had primary TT for multinodular goitre causing mass effect. Five patients had interval completion thyroidectomy following lobectomy where not indicated by guidelines, and 2 due to personal preference on a background of thyroid tumour family history; another patient had interval radiological lymphadenopathy and a recent history of metachronous laryngeal cancer. The remaining 2 patients proceeded to completion thyroidectomy based on preference alone, with age >45 years being their only relative risk factor for recurrence. The rate of surgical management in agreement with the guidelines was not significantly affected by sex ($p = 0.824$), age >45 years ($p = 0.158$), or histological subtype ($p = 0.671$).

Personalised decision-making was recommended following thyroid lobectomy in 32 patients (Table 3); 24 patients had intermediate size tumours (1–4 cm) without risk factors, 4 had microPTC measuring 6–10 mm, while 4 with microPTC <6 mm exhibited multifocal disease. A more aggressive, treatment-driven approach was typically favoured by patients and the multidisciplinary team, with 97% ($n = 31/32$) proceeding to completion thyroid-

ectomy; 1 patient with a unifocal 15-mm papillary TC without risk factors opted for lobectomy alone.

Radioiodine Remnant Ablation

Eighty-two percent of patients (n = 146/178) underwent adjuvant RRA. RRA was more likely to be utilised in patients with node positivity (p = 0.002), lymphovascular invasion (p < 0.001), or extracapsular disease (p = 0.001). RRA utilisation was not affected by sex (p = 0.547), age >45 years (p = 0.203), histological subtype type (p = 0.139), or multifocality (p = 0.067).

In relation to the BTA guidelines, 43% of patients (n = 77) had definitive indications for RRA, while 12% (n = 21) had definitive recommendations against RRA. A personalised decision-making approach was recommended for 45% of patients (n = 80), with 66 of those (83%) progressing to RRA (Table 3). Ninety-seven percent (n = 172/178) of RRA treatment decisions satisfied the 2014 BTA guidelines; 1 patient recommended for RRA due to extrathyroidal extension declined, while the remaining 6 patients received RRA outside of the BTA recommendations. None of these 6 patients had definitive RRA indications; however, 2 had weaker risk factors for recurrence, one with unilateral multifocal microPTC, the other being >45 years old. Agreement with the BTA recommendations was not significantly affected by sex (p = 0.721), age >45 years (p = 0.115), or histological subtype (p = 0.884). Patients with tumours measuring <1 cm were more likely to undergo RRA management differing from the BTA recommendations (i.e., proceeding to RRA) when compared to patients in the 1–4 cm or >4 cm subgroups (p < 0.001).

Discussion

This study assessed the DTC management patterns in an Irish tertiary referral centre, with a focus on patients with intermediate-size, low-risk tumours, where a paucity of evidence prevents guidelines from supporting a definitive therapeutic approach. DTC treatment strategies were largely in agreement with best-practice recommendations, with concordance demonstrated in 94% of surgical and 97% of RRA therapeutic decisions. Where discordance with guidelines was demonstrated, there was a tendency towards overtreatment, with 9 out of 10 surgical and 5 out of 6 RRA treatment decisions resulting in a more aggressive treatment (TT and RRA) where lobectomy and no RRA was recommended. Early studies examining the appropriate surgical management of DTC initially suggested a one size fits all approach with TT, resulting in lower recurrence rates and improved survival for all patients with DTC >1 cm [12, 13]. More recently, recognition of relevant prognosticators has improved risk stratification such that low-risk patients with tumours >1 cm may be treated with more selective and individualised approaches while maintaining improved outcomes [14–17]. This is evidenced by incremental guideline amendments towards more conservative approaches; the 2009 American Thyroid Association (ATA) and the 2006 European Thyroid Cancer Consensus guidelines both previously recommended TT for DTC >1 cm, while the more recent 2014 BTA and 2015 ATA guidelines now suggest lobectomy alone as an option for those with tumours 1–4 cm without risk factors. Emerging evidence also advocates active surveillance for microPTC, with interval growth over 5 years observed in <15% of patients [18, 19]. Improved identification and classification of indolent subtypes further improves risk stratification; the encapsulated follicular variant of papillary TC has recently been re-designated as non-invasive follicular thyroid neoplasm with papillary-like nuclear features, effectively reclassifying it as non-cancerous [20].

Almost half of our cohort was subject to the equivocal BTA guidelines, whereby a personalised decision-making approach was suggested. These patients typically had intermediate-sized tumours without specific recurrence risk factors. Despite large retrospective cohort studies, the infrequency of mortality and disease recurrence events seen in this cohort poses a challenge for the development of definitive evidence-based guidelines. In the absence of specific recommendations, the personalised decision-making approach encourages consideration of factors such as patient preference, age, comorbidity, performance status, ability to engage with contralateral lobe follow-up, and the potential impact of surgical complications. Clinician preference, surgeon complication rates, and tumour parameters tending towards guideline cut-offs (e.g., size approaching 1 or 4 cm) should also be considered. Almost all patients in our cohort who were recommended for a personalised decision-making approach proceeded to the more aggressive options of completion surgery and RRA. The absence of sufficient evidence to support a definitive treatment course in these patients despite multiple large retrospective studies suggests that any benefit gained by this strategy is likely to be small [21]. Furthermore, the improved efficacy of treatments for recurrent DTC is such that the lower risk of recurrence for patients in the personalised decision-making cohort may be acceptable, given the benefit of avoiding a second surgery and exposure to RRA. Benefits gained by the more aggressive options may

be outweighed by both physical and psychological morbidity. The impact of extended patient waiting times, from initial diagnostic imaging and FNAC to thyroid lobectomy, subsequent completion surgery, and onward to RRA, is extensive, resulting in substantial psychological morbidity [22]. Surgical complications are also considerable and include transient (8%) and permanent (2%) hyperparathyroidism, permanent (1%), transient (2%), and diplegic (0.4%) palsies of the recurrent laryngeal nerve, superior thyroid nerve injury (4%), and dysphagia (1%) [23]. There are also financial implications; thyroid lobectomy costs EUR 5,277 per patient in Europe, including presurgical workup, follow-up, and management of complications over 12 months [24], in addition to the lifelong cost of thyroxine replacement, follow-up, and monitoring associated with TT. In New South Wales, Australia, the increased volume of DTC treatments from 2002 to 2012, for a population of 7.5 million, has cost an additional AUD 18,600,000 in surgery-related healthcare expenditure [25].

RRA is also associated with significant side effects. Twenty percent of patients experience nausea, taste and smell impairment, or sialadenitis. More significant complications include impairment of haematopoiesis and gonadal function and increased risk for second primary malignancies, both solid and haematological. A dose of 100 mCi (3.7 GBq) of radioiodine has been estimated to result in an extra 56 malignancies per 10,000 patients over 10 years [24], while Rubino et al. [26] observed up to 30% dose-dependent increased risk for second primary malignancies following RRA.

While DTC has a relatively good prognosis compared to other malignancies, there remains a recurrence risk of 5–30%, and approximately 10% of patients die of this cancer [8, 27–29]. Improved risk stratification is warranted to identify patients at risk of mortality and recurrence. Multiple risk factor assessment tools exist for estimating DTC mortality. Prognostication systems include the AJCC/UICC TNM system (Tumour Nodes Metastases), AMES (Age, Metastases, Extent, Size), MACIS (Metastases, Age, Completeness of resection, Extrathyroidal, Size), EORTC (European Organisation for Research and Treatment of Cancer methodology), and AGES (Age, Grade, Extent, Size) [30, 31]. These prognostication systems, using traditional demographic and staging parameters and focusing predominantly on mortality rather than recurrence risk, have largely failed to guide management in a large subset of patients, as demonstrated by the equivocal BTA 2014 guidelines for low-risk intermediate-size DTC.

Advances in molecular medicine have improved the understanding of DTC carcinogenesis and risk indicators. Molecular markers including gene expression profiles, somatic gene alterations, and circulating biomarkers provide improved indices for diagnosis and prognostication [32]. Alterations in the MAPK and PI3K-AKT major signalling pathways have recently been elucidated as primary pathogenetic events in DTC carcinogenesis [33]. The 2015 ATA guidelines now advocate consideration of $BRAF^{V600E}$ proto-oncogene mutation status, if known, in their modified risk stratification system, although testing is not routinely advocated. The guidelines also acknowledge other gene mutations and rearrangements such as BRAF, TERT, TP53, RAS, or PAX8/PPARγ, although these are not recommended for routine testing [34]. Rather than assessing for individual high-impact mutations, commercially available risk assessment tools analyse panels of mutations, each with smaller odds ratios for recurrence and mortality, but with promising overall accuracy. These include the Afirma Gene Expression Classifier and the ThyroSeq Next-Generation Sequencing panel. These adjuncts have demonstrated encouraging results in the risk stratification of patients with indeterminate FNAC where diagnostic lobectomy is frequently required [35, 36]. In correctly identifying DTC from FNAC samples, ThyroSeq v2.1 reports a sensitivity of 91% (95% CI 79–100) and a specificity of 92% (95% CI 86–98) [36]. This application of molecular risk stratification may help reduce the number of diagnostic thyroid lobectomies undertaken; over one-third of the patients in our cohort had initial Thy3 FNAC results, with three-quarters of these requiring completion thyroidectomy. However, while multiple studies have evaluated the utility of molecular testing in patients with indeterminate thyroid nodules, there is a paucity of research examining the use of such markers in surgical and RRA management decisions after thyroid lobectomy for DTC [32, 37].

Conclusions

DTC management in our cohort exhibited high levels of adherence to internationally recognised best-practice guidelines. Where surgical and RRA therapeutic decisions did not satisfy the guidelines, more aggressive management approaches were usually adopted.

A large proportion of patients are subject to a personalised decision-making approach, owing to a lack of conclusive high-level evidence to guide management. A tendency towards more aggressive surgical and RRA intervention was observed in this group.

These findings highlight the requirement for improved risk stratification to rationalise management strategies and avoid overtreatment of patients who fall into indeterminate-risk treatment groups.

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Statement of Ethics

All patients provided informed written consent, which was ethically approved by the University Hospital Galway research ethics committee.

Disclosure Statement

The authors declare no conflicts of interest.

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RESEARCH

FOXE1 polymorphism rs965513 predisposes to thyroid cancer in a European cohort

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Abstract

Objective: FOXE1 is an intronless gene on chromosome 9 which plays a significant role in thyroid morphogenesis. Mutations in *FOXE1* are associated with thyroid phenotypes including congenital hypothyroidism, thyroid dysgenesis and thyroid cancer. This study aims to investigate the frequency and impact of a SNP (rs965513, G>A) at 9q22.23 in a Western European cohort of patients with differentiated thyroid cancer(DTC), compared to controls.

Design: This is a candidate gene case control study.

Methods: 277 patients with histologically confirmed DTC were recruited from tertiary referral centres in Ireland and France. 309 cancer-free controls were recruited from the community. DNA was extracted from buccal swabs or whole blood of control subjects and patients with DTC. Allelic and genotypic frequencies among patients were compared with controls, to assess the risk for disease conferred by homozygous and heterozygous carriers compared to WT genotypes. Genotyping was performed using Taqman-based PCR.

Results: 277 patients with confirmed DTC and 309 non-cancer controls were genotyped for the variant (rs965513). The frequency of the minor allele among cases was 0.45 compared to 0.34 among controls. The genotypic odds ratio for heterozygotes was 1.66 (CI 1.16–2.39, P =0.00555), increasing to 2.93 (CI 1.70–5.05, P =0.00007) for rare homozygotes. All subjects were in Hardy-Weinberg equilibrium ($\pm\chi^2$, P =0.09, P =0.07 respectively).

Conclusions: This *FOXE1* polymorphism is a low penetrance variant associated with DTC susceptibility in this cohort. The minor allele was identified among patients with thyroid cancer significantly more frequently than controls. An allele dosage effect was observed, with rare homozygous genotypes conferring greater risk than heterozygotes.

Key Words

- differentiated thyroid cancer
- gene polymorphism
- genetics
- risk assessment
- prognostication

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Introduction

Thyroid cancer is the most common endocrine malignancy with an increasing incidence recorded over recent decades in Western countries, including Ireland. Genetic factors play a significant role in the determination of thyroid cancer risk (Amundadottir *et al.* 2004). Epidemiological evidence arising from the Swedish Family-Cancer

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Database of 9.6 million people, estimates that 53% of thyroid cancer risk (all subtypes) is accounted for by genetic rather than environmental factors - the highest among all cancers studied (Czene *et al.* 2002). Medullary thyroid cancer development is associated with point mutations in the RET proto-oncogene on chromosome 10, of which approximately 25% are germline mutations (Accardo *et al.* 2017). While less is known about the sequence variations which confer an increased risk for differentiated thyroid cancer (DTC), it is unlikely that a similar high risk, high penetrance mutation will be found to underlie its pathogenesis. Notwithstanding this, 5–15% of non-medullary thyroid cancer cases are thought to be of familial origin (Khan *et al.* 2010). Multiple mutations, each with relatively smaller effect sizes, together with environmental factors, are more likely to give rise to the majority of DTC (Saenko & Rogounovitch 2018). Numerous genetic alterations have thus far been identified to play a role in thyroid tumourigenesis. Better known examples include mutations in the mitogen-activated protein kinase (MAPK) pathway. The most common of these include BRAF V600E mutations in up to 60% of PTCs, RAS mutations in 15% of PTCs and RET/PTC, ALK or NTRK1 chromosomal rearrangements in 12% of papillary carcinomas (Fagin & Wells 2016, Poller & Glaysher 2017). Other implicated co-existing mutations include TERT promoter, PIK3CA, AKT1 and TP53 mutations (Xing 2013).

Single nucleotide polymorphisms (SNPs) form the basis of many genetic mutations and arise due to nucleotide variation at a single position in a DNA sequence. Genome-wide association studies (GWASs) have identified numerous candidate SNPs which confer increased risk for the development of DTC. Due to the geographical heterogeneity of DTC incidence and pathology, single-track candidate gene studies have subsequently been required to validate candidate SNPs identified by GWAS in various other populations. One such SNP (rs965513, G>A) is located on 9q22.33, at the *FOXE1* gene. The intronless *FOXE1* gene encodes forkhead box protein E1, belonging to the forkhead family of transcription factors and is important for initiation of thyroid organogenesis, cell growth and growth-factor control of thyroid differentiation. *FOXE1* mutations have been associated with phenotypes including congenital hypothyroidism and thyroid dysgenesis. *FOXE1* plays a role in thyroid tumour development, invasion and metastases, is a gene of interest in thyroid cancer research with multiple thyroid cancer risk SNPs near *FOXE1* recently identified (Gudmundsson *et al.* 2009, Chen & Zhang 2018).

This study investigates the impact of a SNP, rs965513 at the *FOXE1* locus, on predisposition to differentiated thyroid cancer in a Western European patient population.

Materials and methods

A casecontrol methodology was utilised to assess the impact of the candidate gene mutation (rs965513, G>A) on DTC risk. Allelic and genotypic frequencies among 277 patients with confirmed DTC were compared with 309 non-cancer controls, to assess DTC risk conferred by homozygous and heterozygous carriers compared to WT genotypes.

A thyroid cancer Biobank was first established at the Discipline of Surgery in the Lambe Institute for Translational Research as part of a multi-centre initiative, comprising clinicopathological data and tissue from patients attending endocrine cancer clinics at tertiary referral centres in the West of Ireland (Galway University Hospital) and in the South of France (Assistance Publique Hôpitaux de Marseille). Patients with histopathologically confirmed DTC, >16 years old, were included. Patients with benign thyroid disease alone, medullary thyroid cancer or familial nonmedullary thyroid cancer were excluded. Patients with any known diagnosis of high-risk germline mutations were also excluded. Controls comprised volunteers >60 years old, without a personal or first degree family history of malignancy, excluding non-melanomatous skin cancer.

All participants provided informed written consent before inclusion. Ethics approval was granted following review by the local institutional Research Ethics Committee.

Demographic and clinicopathological data was recorded at the time of tissue collection from patient self-reporting, electronic histopathology database and patient records. Samples comprised either 10 mL EDTA stabilised whole blood or buccal swab salivary sample (DNA Genotek Oragene 575 collection kit).

DNA was extracted by manual ethanol precipitation. Following extraction, evaluation of the DNA concentration and purity was performed with absorbance spectroscopy (NanoDrop 1000 Spectrophotometer). Samples with a 260:280 nm (A260/280) absorbance ratio ≈ 1.8 were accepted as pure DNA. Real-time PCR and genotyping experiments were undertaken using either the Applied Biosystems StepOnePlus Real-Time PCR System or the 7900HT Fast Real-Time PCR System.

Data recording and statistical analysis were performed using Microsoft Excel 2010 and IBM SPSS v22. Parametric

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tests and means ± standard deviation were utilised for normally distributed data while non-parametric tests and medians with range and interquartile range (IQR) were utilised for non-normally distributed data. Pearson's chi-squared tests were used to compare distributions of categorical variables, while one way ANOVA with Tukey *post-hoc* analysis was utilised for comparisons involving three or more categorical, independent samples. Hardy-Weinberg equilibrium testing of biallelic SNPs was performed using the Pearson's chi-squared test. Chi-squared tests were also used for case-control genetic association analysis.

Results

277 patients with confirmed DTC and 309 non-cancer controls were genotyped for the variant (rs965513). All patients and controls were observed to be in Hardy-Weinberg equilibrium (χ^2, P=0.09, P=0.07 respectively). Patient demographics and clinicopathological characteristics are detailed in Tables 1 and 2 below.

The minor allele frequency was higher in patients with DTC (0.45) compared to controls (0.34) and conferred an increased risk for DTC (per-allele OR 1.61, CI 1.27–2.04, P=0.00008) (Table 3). Both variant genotypes had an increased risk for DTC and demonstrated an allele-dosage association; heterozygous genotypes (AG) had an odds ratio of 1.66 (CI 1.16–2.39, P=0.00555) when compared with wild-types states (GG), increasing to 2.93 (CI 1.70–5.05, P=0.00007) for rare homozygotes (AA).

Subgroup analysis demonstrated statistically significant associations between the both variant genotypes (AG, AA) and DTC, for female gender but not for males. DTC risk association was also present for rare homozygotes, rather than heterozygous states, for tumours >10 mm, but not for patients with sub-centimetre DTC (Figs 1 and 2 & Table 4).

Table 1 Patient and control demographics.

	DTC cases n (%)	Non-cancer controls n (%)
Patients	277 (100%)	309 (100%)
Gender		
Female	208 (75%)	252 (82%)
Male	69 (25%)	57 (18%)
European Caucasian:	255 (92%)	309 (100%)
Other	22 (8%)	0 (0%)
Age, at diagnosis / sampling		
Mean (± S.D.)	46.9 (± 14.95)	73.4 (± 8.68)
Median (range, IQR)	45 (16–84, 23)	74 (60–100, 10)

Table 2 Clinicopathological patient and tumour characteristics.

	DTC cases n (%)
Patients	277 (100)
Tumour size	
Mean (± S.D.)	26 (± 18.05)
Median (range, IQR) (mm)	22 (1–110, 22)
Papillary	236 (85%)
Follicular	41 (15%)
Multi-focal	89 (32%)
Node positive	56 (20%)
Distant metastatic disease	8 (3%)
Lymphovascular invasion	44 (16%)
Personal history of other cancer[1]	25 (9%)
Family history of DTC[2]	23 (8%)

[1]Excluding non-melanomatous skin cancer; [2]First degree relatives only.

Variations in MAF and genotypic odds ratios were also observed between Irish and French subgroups, with the minor allele observed more frequently among patients recruited in Ireland, in addition to conferring a greater risk for DTC compared with the French cohort (Table 5). In addition to higher genotypic odds ratios among the Irish subgroup, an increased per-allele ratio of 1.84 (95% CI 1.40–2.41, P=0.00001) was also observed, compared with a per-allele odds ratio of 1.31 among the French subgroup (95% CI 0.95–1.80, P= 0.09678).

Discussion

Molecular biomarkers have the potential to improve our estimation of DTC risk, and possess a number of advantages over conventional biomarkers. In particular, germline mutations remain stable irrespective of patient, disease or treatment factors, are measurable at or before disease onset, and are amenable to high-throughput assays, now commonly available in modern laboratories. In light of DTC-specific 5 year survival rates in excess of 95%, and increasing incidence rates worldwide, clinicians are challenged to avoid overdiagnosing and overtreating potentially low grade, indolent thyroid lesions, while appropriately managing those requiring more aggressive treatment. Our results demonstrate an association between the rs965513 SNP in the *FOXE1* gene, and an increased risk for development of DTC. Furthermore, we demonstrate an association with disease phenotype and therefore, this marker may confer prognostic benefits to improve DTC risk stratification.

The rs965513 SNP was initially identified as a risk factor for DTC (OR=1.75, P<0.001) in an Icelandic genome-wide association (Gudmundsson *et al.* 2009). A number

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Table 3 Genotype frequencies, genotypic odds ratios, and minor allele frequencies in DTC for all cases vs controls.

rs965513 (GA)	**Common homozygote** (GG), **n** (%)	**Heterozygote** (AG), **n** (%)	**Rare homozygote** (AA), **n** (%)	**Minor allele frequencies**
Cancers	77 (28%)	151 (55%)	49 (17%)	0.45
Controls	129 (42%)	152 (49%)	28 (9%)	0.34
Odds ratio (95% CI)		1.66 (1.16–2.39)	2.93 (1.7–5.05)	
Significance (*P*)		0.00555	0.00007	

of studies have subsequently examined the association in various populations, including two large meta-analyses, which both supported an association between rs965513 and DTC susceptibility, but reported widespread heterogeneity between study methodologies and outcomes, in particular among studies examining Ccaucasian populations (Wang *et al.* 2016, Chen & Zhang 2018). Furthermore, a number of studies have also reported an absence of association between DTC and the risk allele (Denny *et al.* 2011, Kang *et al.* 2014).

To date, just one study has examined the association between rs965513 and DTC susceptibility in a UK population, while no published work has included data from Irish or French cohorts (Jones *et al.* 2012). Incidence of thyroid cancer varies considerably between populations. Amongst European nations, the highest rates of thyroid cancer are seen in Lithuania (15.5/100,000), Italy (13.5/100,000) and France (11.7/100,000) (European Network of Cancer Registries 2017). The incidence in Ireland is 5.8/100,000 (Irish Cancer Society 2017). Furthermore, the rate of change in incidence also varies geographically; for example in France incidence has risen from 3.4 to 11.7 per 100,000 from 1983 to 2002, while in Sweden incidence has remained more stable, reported as 2.4/100,000 in 1958 and 3.5/100,000 in 2002 (Brito *et al.* 2014). In Ireland, incidence has increased three-fold, from 1.9/100,000 in 1994 to 5.8/100,000 in 2017 (National Cancer Registry Ireland 2012, Irish Cancer Society 2017). While the cause of such geographical variation in incidence may be explained by differences in population genetics, other factors likely play a role; these include variable exposure to environmental ionising radiation, and in particular, variations in the prevailing approach to the diagnosis and management of thyroid disease and nodules, with more aggressive investigation, and perhaps over-diagnosis, of thyroid disease playing a role. Despite this, such significant geographical variation in thyroid cancer epidemiology, and reported population-based heterogeneity in DTC risk associated with rs965513, further validation of this risk allele is warranted.

An allele dose association between the rs965513 variant allele and DTC was demonstrated in this study, with odds ratios of 1.66 and 2.93 for heterozygous and rare homozygous states respectively and a per-allele odds ratio

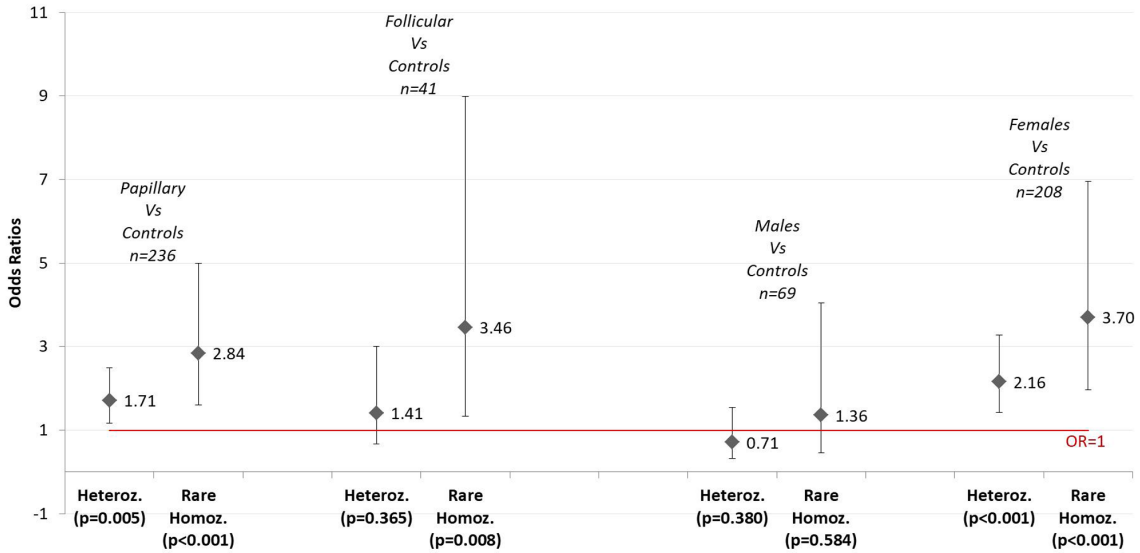

Figure 1
Genotypic odds ratios for sub-groups vs controls comparing rare homozygotes and heterozygotes with wild-types states: histological subtypes and gender.

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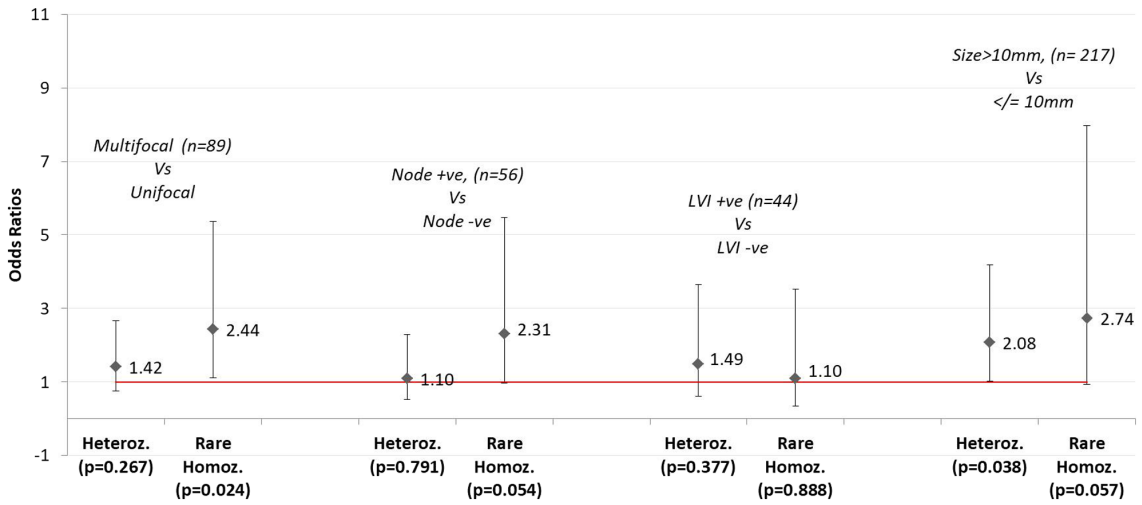

Figure 2
Genotypic odds ratios comparing rare homozygotes and heterozygotes with wild-types states for multi-focality, nodal status, lymphovascular invasion status and tumour size.

of 1.61 compared to controls. This compares to per-allele odds ratios of 1.99, 1.75 and 1.82 reported in UK (n=781), Icelandic (n=192) and Polish (n=1795) cohorts respectively, while one Portuguese study reported a per-allele odds ratio of 2.81 in a group of 80 patients with sporadic non-medullary thyroid cancer (Gudmundsson *et al.* 2009, Jones *et al.* 2012, Tomaz *et al.* 2012, Liyanarachchi *et al.* 2013). The frequency of the minor allele was 0.45 in our cohort which compares to 0.49, 0.49, 0.46 and 0.61 in the UK, Icelandic, Polish and Portuguese cohorts respectively. Variations in risk between populations and subgroups may arise for various reasons including true biological differences, variability in modifiable environmental risk factors or differences in sample size and study methodology.

This study recruited patients from tertiary centres in France (n=109) and Ireland (n=168). The risk allele was more prevalent in Irish than French subjects (MAF Irish 0.48, French 0.4 and Control group 0.34), with associated higher per-allele odds ratios evident in the Irish subgroup, compared with the French (OR=1.31, P=0.096 vs OR=1.84, P=0.00001 respectively). Presence of the rare homozygous genotype among French subjects conferred an odds ratio for DTC of 1.94 (P=0.066). Rare homozygous genotypes in the Irish subgroup demonstrated an odds ratio of 3.9 (P<0.00001); an almost four fold increase in DTC susceptibility in the presence of two variant alleles at the locus represents a significant risk factor for DTC. While our results suggest its impact on DTC risk may be more significant in Irish rather than French patients, it is notable that only 15% (n=16/109) of French subjects were rare homozygous carriers, compared to 20% of Irish subjects (n=33/168); however, assessment of a larger French cohort may also yield a statistically significant odds ratio for DTC. Perhaps the increased rates of homozygous carriers and the higher MAF amongst Irish DTC patients may be attributable to the relative homogeneity of Irish population genetics, arising due to their island location, geographically isolated from continental Europe, historically low rates of inward migration and the relatively late human colonisation of Ireland compared to the rest of Europe (O'Dushlaine *et al.* 2010). Furthermore, a limitation of this study is the absence of French subjects included in the control arm, which may limit conclusions that can be drawn from subgroup analysis specific to the French subgroup. The observed difference in MAF between populations merits further investigation in a larger study with controls of matched ethnicity.

The minor allele frequency measured 0.46 in female cases and 0.32 in female controls, compared to a narrower differential between male cases and controls of 0.41 and 0.39 respectively. Our cohort exhibited an allele dose gender association, with female risk genotypes carrying a higher risk for DTC compared to female controls, while no significant association was demonstrated between male variant genotypes vs male controls. Heterozygous females carried an odds ratio of 2.6 (95% CI 1.42–3.27, P=0.0003), rising to 3.7 (95% CI 1.96–6.96, P=0.00003) for rare homozygous females. In contrast, equivalent odds ratios for male variant genotypes vs controls measured 0.71 and 1.36 (P=ns) for heterozygotes and rare homozygous carriers respectively. The gender distribution of DTC gives rise to insufficient power to adequately assess the variant effect in our male cases (n=69, 25%). However, given the gender

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Table 4 Subgroup analysis: genotypic odds ratios and minor allele frequencies.

SNP G>A	**Wild-type** (GG)	**Heterozygote** (AG)	**Rare homozygote** (AA)	**Minor allele frequency**
Papillary cases	65	131	40	0.48
All controls	129	152	28	0.34
Odds ratio (CI)		1.71 (95% CI 1.17–2.5)	2.84 (95% CI 1.61–5.0)	
P value		0.00531210	0.00024632	
Follicular cases	12	20	9	0.46
All controls	129	152	28	0.34
Odds ratio (CI)		1.41 (95% CI 0.67–3.0)	3.46 (95% CI 1.33–8.99)	
P value		0.36518017	0.00797090	
Male cases	26	30	13	0.41
Male controls	19	31	7	0.39
Odds ratio (CI)		0.71 (95% CI 0.33–1.54)	1.36 (95% CI 0.46–4.05)	
P value		0.38021705	0.58388242	
Female cases	51	121	36	0.46
Female controls	110	121	21	0.32
Odds ratio (CI)		2.16 (95% CI 1.42–3.27)	3.7 (95% CI 1.96–6.96)	
P value		0.00026969	0.00003028	
Multi-focal cases	19	49	21	0.51
Unifocal cases	53	96	24	0.42
Odds ratio (CI)		1.42 (95% CI 0.76–2.67)	2.44 (95% CI 1.11–5.36)	
P value		0.26740704	0.02448424	
Node +ve cases	13	28	15	0.52
Node -ve cases	60	117	30	0.43
Odds ratio (CI)		1.1 (95% CI 0.53–2.29)	2.31 (95% CI 0.97–5.47)	
P value		0.79133678	0.05408739	
LVI* cases	9	28	7	0.48
Non LVI cases	24	50	17	0.46
Odds ratio (CI)		1.49 (95% CI 0.61–3.65)	1.1 (95% CI 0.34–3.53)	
P value		0.37714114	0.88753708	
>10mm cases	54	124	39	0.47
≤10mm cases	19	21	5	0.34
Odds ratio (CI)		2.08 (95% CI 1.03–4.18)	2.74 (95% CI 0.94–7.98)	
P value		0.03766692	0.05708887	

differential in MAF, in addition to significant odds ratios observed for female genotypes, a gender variance is evident. Of the above mentioned studies, Liyanarachchi *et al* comment explicitly on gender specific risk conferred by the variant; of their 1795 Polish DTC cases, no difference in MAF or DTC risk was evident between genders. Similarly, Gudmundsson *et al.* reported an absence of significant MAF or rs965513 risk difference between genders in their

Table 5 Genotype frequencies, genotypic odds ratios, and minor allele frequencies in DTC for French and Irish populations vs controls.

rs965513 (G → A)	**Common homozygote** (GG), **n**(%)	**Heterozygote** (AG), **n**(%)	**Rare homozygote** (AA), **n**(%)	**Minor allele frequencies**
Controls	129 (42%)	152 (49%)	28 (9%)	0.34
Irish cases	39 (23%)	96 (57%)	33 (20%)	0.48
Odds ratio (95% CI)		2.09 (1.35–3.24) *P* = 0.00093	3.898 (2.10–7.23) *P*< 0.00001	
French cases	38 (35%)	55 (50%)	16 (15%)	0.40
Odds ratio (95% CI)		1.228 (0.76–1.98) *P* = 0.39606	1.94 (0.95–3.96) *P* = 0.06571	

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Icelandic cohort. While our cohort does exhibit an allele dose gender association, these results should be interpreted with caution given that secondary analyses with small sample sizes are at risk of Type 1 error, particularly in light of the above mentioned larger studies provided conflicting results.

In addition to contributing to risk estimation for the initial development of DTC, germline SNPs identified by GWAS or candidate gene studies may also impact the clinical course of DTC after diagnosis, and estimate risk for the development of particular pathological characteristics. Our data suggests that rs965513 is associated with tumours >10mm and multifocality. A recent review identifies rs965513 as being associated with increased DTC tumour size and extra-thyroidal extension (Jendrzejewski *et al.* 2019). Other germline mutations have also been linked to additional pathological characteristics including nodal disease burden, metastatic disease and disease-specific mortality (Jendrzejewski *et al.* 2019). It follows that testing for rs965513 as part of a multigene mutational panel may not only estimate the likelihood of DTC occurrence, but also aid in stratifying patients in terms of locoregional, metastatic or recurrent disease risk, thereby informing decisions around treatment strategies such as the need for completion thyroidectomy, nodal dissection, radioiodine remnant ablation or TSH suppression. Furthermore, genotyping patients for rs965513 potentially adds valuable information for the management of cases that fall into an indeterminate or 'individualised decision making' group as described in the British Thyroid Association guidelines, which are currently based only on standard clinicopathologic features (Perros *et al.* 2014).

Our data is concordant with findings of published GWAS and meta-analyses confirming that rs965513 is a low penetrance variant associated with DTC susceptibility. We conclude that assessment of rs965513 may be considered as part of a gene mutation panel to improve DTC risk stratification in patients with this common malignancy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Statement of ethics

All subjects involved in this study provided written informed consent for inclusion and the study protocol was approved by the Institutional Ethics Review Board at Galway University Hospital (Ref 45/05 C.A.151).

Author contribution statement

AL, MK and FS were the supervising physicians, involved in planning, and supervision of the work. PO, TM, CG, MB and DQ undertook collection of patient samples. PO processed samples and data, performed experiments, and drafted the manuscript. All authors considered the outcomes and reviewed the manuscript.

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