

MOLECULAR GENETIC INVESTIGATION OF MEDULLARY THYROID CANCER

by

JOEL ANTHONY SMITH



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Abstract

Introduction

Most familial MTC is caused by a germline mutation of the RET proto-oncogene. Rare families exist with predisposition to MTC in whom no RET mutation has been identified. Identification of novel candidate genes within such families may inform the molecular behaviour of more common, sporadic disease. Whole exome sequencing (WES) enables all protein coding regions of the genome to be sequenced in parallel; a novel paradigm for MTC gene discovery.

Methods

Patients with MTC were recruited through internationally developed collaborations. WES was completed in three generations of the index family. Germline and tumour DNA were analysed for conformational mutations. *In vitro* functional analysis of candidate genes was completed to unpick biological pathways.

Results

Over 20,000 mutations were screened. A frameshift mutation in the oestrogen receptor 2 gene (ESR2) has been identified with familial segregation. Further alterations in ESR2 have been identified in germline DNA from a patient with young onset sporadic disease and tumour DNA from sporadic MTC. The functional protein of the ESR2 gene binds to a response element in the upstream pathway of the RET gene, controlling transcription. *In-vitro* studies show that ESR2 mutants lead to null proteins and up-regulation of RET at mRNA and protein levels. Further, loss of ESR2 protein is observed in patients with germline ESR2 mutations.

Conclusions

This study establishes a novel method of gene predisposition identification in the context of MTC. As well as the potential for a genetic test, and as a prognostic biomarker, the on-going functional work may elucidate targets for novel therapies that may include pre-existing anti-oestrogens.

Dedication

To Cara, Barnaby and Alexa

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Professor Eamon Maher	Professor of Medical and Molecular Genetics, Cambridge
Professor Chris McCabe	Professor Endocrinology, UoB
Mr John Watkinson	Consultant ENT surgeon, UHB
Dr Martin Read	Post doctoral researcher, UoB
Dr Rachel Brown	Consultant Pathologist, UHB
Jon Hoffman	Research coordinator, BWH
Dr Naomi Wake	Post doctoral researcher, UoB
Dr Rachael Watkins	Post doctoral researcher, UoB
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Collaborators

Mrs Jo Grey	CEO AMEND charity
Mr T Kurzawinski	Consultant Surgeon, GOS
Prof S Sidhu	Professor of Surgery, Sydney
Dr Gudwara,	Post Doctoral Researcher, Sydney
Prof B Robinson	Professor of Genetics, Sydney
Prof M Robledo,	Spanish National Cancer Research Centre
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List of abbreviations

AHNS	American Head and Neck Society
ATA	American Thyroid Association
BCA	Bicinchoninic acid
BJS	British Journal of Surgery
BSA	Bovine Serum Albumin
BTA	British Thyroid Association
BTF	Butterfly thyroid Foundation
BWH	Birmingham Women's Hospital
cDNA	Complimentary Deoxyribonucleic acid
CCH	C Cell Hyperplasia
CGU	Clinical Genetics Unit
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DCT	Distal Convoluted Tubule
DIT	Di-iodo-tyrosine
DMEM	Dulbecco Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Nucleotide triphosphates
E2	Oestrogen
ER α	Oestrogen receptor alpha
ER β	Oestrogen receptor beta
ERE	Oestrogen response element
ESES	European Society Endocrine Surgeons
ESR1	Oestrogen Receptor 1
ESR2	Oestrogen Receptor 2
FBS	Fetal Bovine Serum
FMTC	Familial Medullary Thyroid Cancer
G	Gravity
GOS	Great Ormond Street
HRP	Horseradish peroxidase
LB	Luria Both
MEN1	Multiple Endocrine Neoplasia Type 1
MEN2A/B	Multiple Endocrine Neoplasia Type 2
mRNA	Messenger ribonucleic acid
MTC	Medullary Thyroid Cancer
PCR	Polymerase chain reaction
PTC	Papillary thyroid carcinoma
PTH	Parathyroid Hormone
RAF	Serine/threonine-protein kinase
RAS	Rat sarcoma viral oncogene
RB	Retinoblastoma
RET	REarranged during Transfection
RIPA	Radioimmunoprecipitation assay
RLN	Recurrent laryngeal nerve
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

siRNA	Short interfering RNA
SHC	Scr homology 2 domain
SOC	Super optimised culture
T3	Tri-iodothyronine
T4	Tetraiodothyronine
TBS-T	Tris buffered saline with tween
TEMED	Tetramethylethylenediamine
Tg	Thyroglobulin
TRH	Thyrotrophin releasing hormone
TSH	Thyroid Stimulating Hormone
UK	United Kingdom
US	United States
UHB	University Hospital Birmingham
UoB	University of Birmingham
v/v	volume to volume ratio
VO	Vector only
w/v	Weight to volume ratio
WES	Whole Exome Sequencing
WHO	World Health Organisation
WGS	Whole Genome Sequencing

Chapter 1.

General Introduction

1.1 The Thyroid Gland: Anatomy, physiology and embryology

This section highlights the salient anatomical, embryological and physiological aspects of the thyroid gland and their relation to thyroid malignancy. This section includes only the features relevant to this project and as such is neither a complete review of the topics nor an exhaustive reference. The aim is to highlight the challenges posed by thyroid cancer in general and the specific issues that make medullary thyroid cancer (MTC) more difficult to treat and manage both in the clinic and in the operating room. This project has a surgical background, as it is the challenges faced when working with often young patients with recurrent or residual MTC, that have driven this project. More and targeted research is required in this area such that outcomes from this disease are ultimately improved.

1.1.1 The surgical anatomy of the thyroid gland limits the extent to which surgery can be curative.

Surgery is the only treatment in MTC that improves survival (Kloos et al., 2009). Surgery is both the primary treatment modality of choice and the choice for recurrent disease (Watkinson and Gilbert, 2012). The limitations of surgery are defined by the local anatomy and by the disease process. It is the anatomical position of the gland leads to difficulties in management, in particular when considering residual and recurrence disease (Watkinson and Gilbert, 2012). Patients with locally advanced and recurrent disease are often faced in the clinical setting and are potentially the most difficult to manage (Hardman et al., 2014). Recurrent surgery to the in the neck carries significant

morbidity, and surgery is only an option when disease recurs locally. There is little in the way of effective treatment options for distant recurrence and although novel therapies to date have shown some improvement in disease response, no improvement in life expectancy has been demonstrated (Wells et al., 2012). There is a need for a better understanding of the biology of MTC so that effective adjuncts or alternatives to surgery can be developed.

Macroscopically, the thyroid gland consists of two distinct lobes, left and right joined by the thyroid isthmus, a narrow strip of tissue overlying the trachea (Gray Henry, 1974). Occasionally, a pyramidal lobe is present, representing an upward extension of thyroid tissue along the thyroglossal duct, containing a variable amount of thyroid tissue. This can represent the site of a primary malignancy or an area of recurrence (Rossi et al., 2014)(Moore Keith, Dasley Arthur, Agur Anne, 2013). The thyroid lobes sit on either side of the trachea, deep the strap muscles of the neck and lying medial to the vascular compartment containing the carotid artery and internal jugular veins on either side (Gray Henry, 1974). The isthmus joins the lobes together and overlies the trachea crossing typically at the second to fourth tracheal ring (Watkinson and Gilbert, 2012)(Gleeson and Clarke, 2008) (Figure 1).

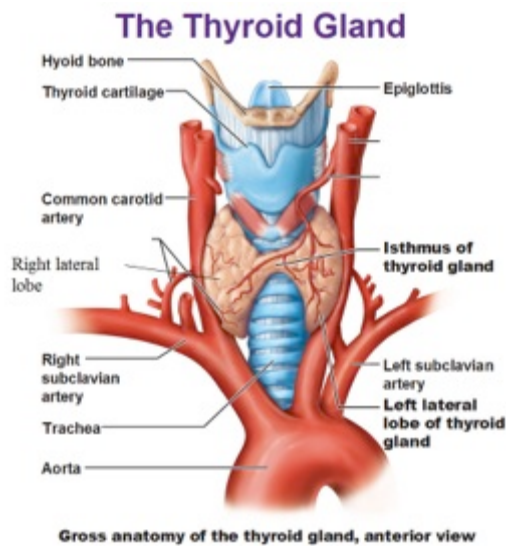


Figure 1. Gross anatomy of the thyroid gland and its relation to the upper aero-digestive tract and major vessels.

Stage at presentation		10 year survival
Stage I	pT1, N0, M0	100%
Stage II	pT2, N0, M0 pT3, N0, M0 pT4, N0, M0	93%
Stage III	Any pT, N1, M0	71%
Stage IV	Any pT, any N, M1	21%

Figure 2. Staging and 10 year survival of medullary thyroid cancer (T = tumour, N = lymph nodal status, M = metastasis) (Modigliani et al., 1998).

The depth of the gland within the neck and the fact that the majority of thyroid tissue is in the lower pole means that significant occult swelling can occur without symptoms and malignant change in the gland can go un-noticed until local disease is relatively advanced (Cooper et al., 2009). As an illustration, the proband in the index family in this project (discussed in Chapter 3) was identified incidentally after presenting to accident and emergency after a minor road traffic accident. Further, my recent review of over 300 patients with

thyroid cancer presenting to University Hospital Birmingham (UHB), >90% of cases had primary tumours >2cm in size at presentation (T2-T4) and the majority of small tumours were discovered incidentally and without symptom (Smith et al., 2014). With an absence of symptomatic disease in early stages, disease stage at presentation can therefore often be advanced, with 50% of MTC presenting stage III or above (Kloos et al., 2009). Ten-year survival for stages 1-4 disease are 100%, 93%, 71% and 21% respectively with no trend toward earlier stage at presentation (Modigliani et al., 1998). This has a significant impact on prognosis in MTC (Kloos et al., 2009). Further, in advanced local disease, involvement of the vascular compartment and upper aero-digestive tract, in particular carotid artery encasement, may render disease inoperable and as such incurable (Watkinson and Gilbert, 2012).

The proximity of the thyroid gland to other structures may affect the way in which thyroid cancer presents. The superior poles of the lobes on either side receive the upper poles vessels including the superior thyroid artery and vein and the superior laryngeal nerve is in close proximity supplying the cricothyroid muscle which provides the superior poles medial relation (Sinnatamby, Chummy S, 2006). The inferior pole is more lobular in shape and receives blood from the inferior thyroid artery with venous drainage via the middle and inferior thyroid veins (Figure 1) (Moore Keith, Dasley Arthur, Agur Anne, 2013). The recurrent laryngeal nerve, a branch of the vagus nerve, lies deep to the gland running in the tracheoesophageal groove on the left and more laterally on the right and is intimately related to the inferior thyroid artery running over, under or between its branches (Haller, Iwanik and

Shen, 2012). Malignant disease that spreads beyond the thyroid capsule can directly invade the laryngeal nerves leading to dysphonia, or airway compromise (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014a). Further, when surgical removal of the gland for cancer treatment is warranted, damage to the recurrent laryngeal nerves can lead to airway compromise and death due to paralysis of the laryngeal musculature (Randolph et al., 2011). Often therefore, microscopic disease has to be left behind in order to reduce morbidity and preserve airway function (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014a). Partial tracheal resection or indeed laryngeal and tracheal resection may be warranted in such cases and will have significant impact on a patient's quality of life (Bergamaschi et al., 1998). Surgery may be considered in these situations as part of a palliative procedure to reduce the likelihood of asphyxiation or major haemorrhage (Akslen et al., 1991). Surgery may, on the other hand, be limited where there is significant metastatic disease, with a morbidity sparing approach being adopted (Watkinson and Gilbert, 2012). These issues arise more commonly in patients with MTC as there are few other treatments we can offer for recurrent disease (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014a). Surgery is often combined with external beam radiotherapy in these situations but treatment is likely to be with palliative intent only, adding weight to the need for further therapies being developed for patients with MTC.

The thyroid gland has close anatomical relations to the parathyroid glands, with two superior and two inferior parathyroid glands sitting in close proximity to the posterior surface of the thyroid lobe (Gray Henry, 1974). As the

parathyroid glands are involved in calcium homeostasis by the production of parathyroid hormone in response to low levels of serum ionised calcium, inadvertent damage or removal during surgery can lead to postoperative hypocalcaemia and, in severe cases, tetany with cardiac dysrhythmia and death (Sturniolo et al., 2000). Parathyroid disease in the form of adenomas and hyperplasia may also be a feature of multiple endocrine neoplasia (MEN) type 1 and 2 respectively, with type MEN2 having a consistent association with medullary thyroid cancer (Brandi et al., 2001).

1.1.2 Thyroid cancers metastasise to loco regional lymphatics

The lymphatic drainage of the thyroid gland has important implications for MTC disease presentation and spread. Primary spread of malignant disease of the thyroid gland, regardless of the tumour type, will be to the local lymphatics. Microscopically and macroscopically, nodes will be involved in 20-50% of cases at presentation (Nam-Goong et al., 2004), with extensive neck nodal disease at presentation carrying a poorer prognosis (Nixon et al., 2014). This highlights the rich lymphatic drainage of the thyroid gland as well as early lymphatic involvement being a characteristic feature of thyroid cancer (Grebe and Hay, 1996). Furthermore, well-differentiated thyroid cancer and MTC can present primarily with lymphatic metastases with no clinical or radiological evidence of a primary lesion. In such cases, a micro-carcinoma (<10mm) is often discovered on subsequent histological examination (Smith et al., 2014).

Lymphatic metastasis within the neck is considered in levels and tumour specific patterns of spread are observed within the head and neck region (Watkinson and Gilbert, 2012). The thyroid gland sits within level six of the

neck and it is to level six that thyroid cancers most often metastasise (Robbins et al., 2008). Level six is the anatomical region in the central neck between the carotids on either side and from the hyoid to clavicles (Figure 3). When treating patients with known thyroid cancer, an ipsilateral level six lymph node dissection will often be performed as part of the thyroidectomy in cases where no nodal disease is macroscopically apparent (N0 Neck) (Kloos et al., 2009).

Lymph node metastasis in the central compartment are underestimated both clinically and radiologically (Moley and DeBenedetti, 1999). With a lack of adjuncts to surgery for the treatment of MTC, central compartment (level six) clearance is advocated in the absence of macroscopic disease, although this approach is not advocated in prophylactic surgery (Pelizzo et al., 2007). Lateral spread of disease typically affects levels IIA to level VB, although theoretically, any group can be involved (Figure 3). If lateral nodes are affected, a clearance of these levels is recommended along with central nodal clearance (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014b). Primary surgery where possible is advocated as re-operative central node clearance is associated with higher rates of complications including recurrent laryngeal nerve palsy and hypocalcaemia (Weber et al., 2001).

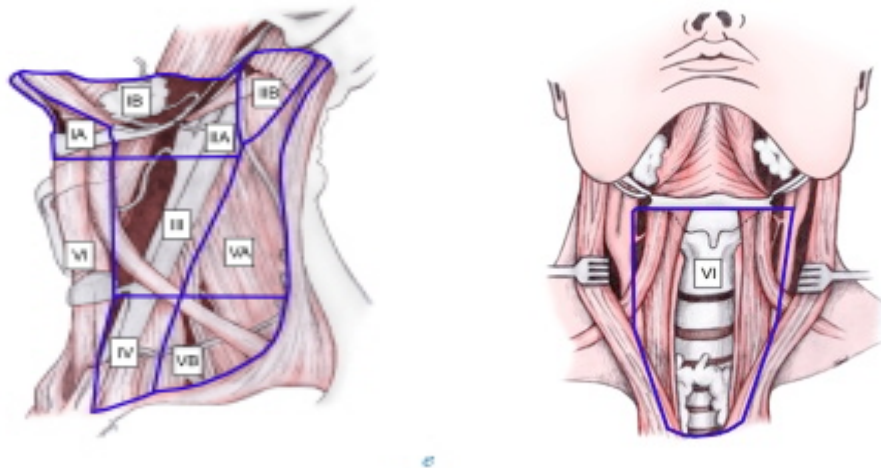


Figure 3. Neck nodal levels with regard to the spread of malignancies within the neck. (Watkinson and Gilbert, 2012).

In the absence of macroscopic nodal disease involving levels II to V, (the 'lateral' compartment) most would advocate a conservative approach in the presence of MTC with re-exploration as clinically dictated (Robbins et al., 2008). In well-differentiated disease, such as PTC, microscopic disease is likely to be well treated by radioactive Iodine (^{131}I) therapy and as such the pressure to surgically remove all disease in the lymphatics is diminished (Leboulleux et al., 2005). As a surgical disease not responsive to ^{131}I therapy, in MTC, both microscopic and macroscopic disease must be removed if long-term survival and disease free recurrence is to be achieved (Kloos et al., 2009). To this end, and as evidenced by our own cohort, patients with MTC often require multiple surgical procedures (Chapter 3).

1.1.3 The thyroid is an endocrine gland consisting of cells with different function.

The physiological differences between thyrocytes and C cells account for some of the disparity in treatment outcomes. The thyroid gland is an endocrine organ structurally organised at a cellular level and consisting of cells with differing physiological function. The primary function of the thyroid gland is the production and release of thyroxine, vital for growth, differentiation and metabolism within most tissues (Oppenheimer et al., 1987). The primary and most abundant cell is the thyrocyte or follicular cell; cuboidal epithelial cells which are organised around structural units known as follicles (Yen, 2001). The follicle itself is located at the apical end of the thyrocyte and forms a storage reservoir for organified iodine and thyroglobulin. The thyroid gland also contains C cells, or para-follicular cells, lymphocytes, blood vessels and lymphatic channels. The primary function of the thyrocyte is in the uptake, organification and storage of iodine required in the synthesis of thyroxine (Kopp, 2001). The uptake of iodine and release of thyroxine are controlled by thyroid stimulating hormone (TSH) which in turn is regulated by thyroid releasing hormone (TRH) (Vassart and Dumont, 1992). TSH and TRH are released from the pituitary and hypothalamus respectively under the control of serum thyroxine as part of a negative feedback loop (Figure 4) (Shupnik, Ridgway and Chin, 1989). TSH acts via the TSH receptor on the basal membrane of the thyrocyte which sets off an intracellular cascade leading to activation of thyro peroxidase and secretion of organified iodine as mono and di-iodothyronine at the basilar membrane where it stored in the thyroid follicle.

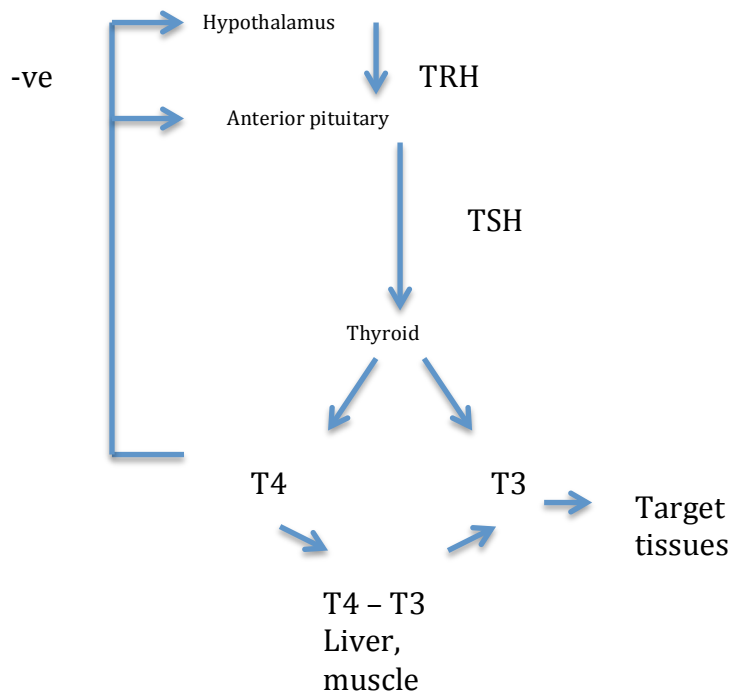


Figure 4. Hypothalamic-pituitary–thyroid axis.

In contrast, C-cells secrete calcitonin, a 32 polypeptide hormone encoded by the CALCA gene and involved in calcium homestasis (Le Moullec et al., 1984)(Copp and Cheney, 1962). Calcitonin is secreted in response to increased blood levels of ionised calcium and as such plays a role in calcium homeostasis, promoting osteoblastic activity, limiting the activity of osteoclasts and therefore calcium resorption from bone and increasing calcium loss in the kidney by decreasing resorption in the distal convoluted tubule (DCT), with a further reduction in intestinal calcium absorption from the gut (Guyétant et al., 2003). Interestingly, within the context of this study, an association between oestrogen and calcitonin secretion has long been recognised with falling levels of calcitonin in post-menopausal women implicated in loss of bone density and osteoporosis (Stevenson, 1982).

C cells in humans are found within the thyroid and are part of the ultimobranchial complex in other animals (Stevenson, 1982). The role of C cells and calcitonin in calcium homeostasis in humans is somewhat limited; a fact highlighted by the lack of abnormality in calcium homeostasis seen in patients who have undergone total thyroidectomy. Although the effects of calcitonin on calcium homeostasis mirror that of PTH, it is far less potent in effecting blood calcium levels (Breimer, MacIntyre and Zaidi, 1988). Other factors including, calcium resorption and loss in the kidney clearly predominate. Serum calcitonin measurements form the basis of clinical tests for patients with suspected primary MTC as well as biochemical monitoring of patients following treatment. Serum calcitonin levels are an accurate marker of residual disease and disease recurrence in MTC. Rising serum calcitonin correlates well with recurrent disease with calcitonin doubling times being a marker of disease requiring investigation and possibly intervention (Costante et al., 2007).

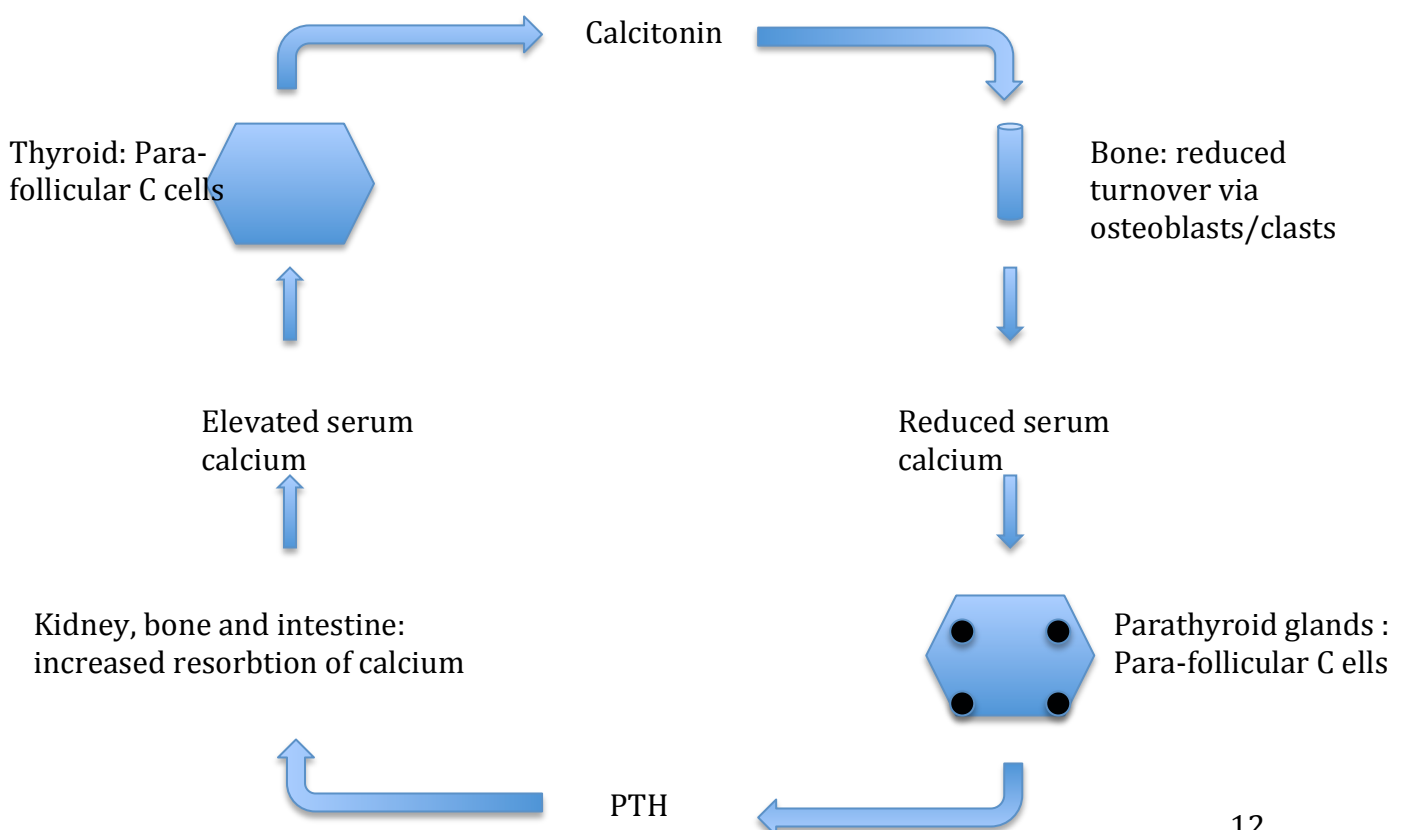


Figure 5. Calcium homeostasis and regulation by parathyroid hormone (PTH) and calcitonin

C-cells can be stimulated to release calcitonin by gastrin and pentagastrin (Escalada et al., 1993). Both basal calcitonin and pentagastrin-stimulated calcitonin can be used as an investigative tool for patients with suspected MTC or C Cell hyperplasia (CCH: C-cells can undergo hyperplasia (increased cell number), a recognised premalignant change) as a guide to further investigation or treatment in the form total thyroidectomy (Milone et al., 2010). It can be of particular use in patients with a positive family history of MTC in whom no germline RET abnormality has been detected (see Chapter 3) and was used within our index family as a guide to treatment decision making with respect to prophylactic thyroidectomy in the offspring of the proband (Herrmann et al., 2010). As test of CCH however, pentagastrin stimulation is not totally reliable and indeed proved a poor marker of histopathological outcome in our index family (see Chapter 3). The presence of CCH in pentagastrin positive patients with benign thyroid (nodular and thyrotoxic) disease is reported as high as 50% (Scheuba et al., 2000). In addition, antibodies to calcitonin can be used to highlight C cells on histological slides, and it is worth noting that both C-cells and CCH are more common in men than women (Bléchet et al., 2007).

1.1.4 The embryology of the thyroid gland is important when considering the functions and dysfunction of cells of which it constitutes

Medullary thyroid cancer (MTC) is a tumour derived from neural crest para-follicular C-cells which have a distinct embryological and developmental origin to follicular cells and are dispersed throughout the thyroid gland (Kloos et al., 2009). Thyroid tissue, consisting of thyrocytes surrounding follicles of colloid, develops in the foramen caecum of the tongue base and descends through the anterior neck during embryogenesis to lie in the lower neck (Sadler, 2010). The tract through which the gland descends (thyroglossal duct) can fail to close resulting in a thyroglossal duct cyst or a thyroid tissue remnant within the duct (McCoul and de Vries, 2009), with such remnants are more prone to malignant change. In addition there may be failed migration of the gland along the duct with the entire thyroid gland can be found from anywhere between the tongue base and the neck (Organ and Organ, 2000).

By contrast, the embryological origin of C cells is the neural crest, pleuropotent cells that originate from the dorsal and later aspects of the neural tube (Sadler, 2010). They possess the potential to both migrate and differentiate into a wide range of tissue types with myriad function. In the head and neck region, they migrate into the branchial arches where they form branchial arch structures such as the bones and cartilage of the facial skeleton, mandible and middle ear (Fagman and Nilsson, 2010). Lower in the neck, and in particular in the fourth branchial arch, neural crest cells migrate

with the ultimobranchial body, the final embryological inclusion of the ventral fourth arch (Kameda et al., 2013). The ultimobranchial body containing the neural crest inclusions then merges with the descending thyroid gland and the C cells are dispersed with it (Adams and Bronner-Fraser, 2009). Ultimobranchial dispersions can be seen quite clearly in the immunohistochemical studies (Chapter 7). The ultimobranchial body also forms the thymic structures which migrate into the upper mediastinum (Fagman and Nilsson, 2010). The differing embryological origin of C cells and primary thyrocytes has implications when studying models of cellular dysfunction. In particular, and as discussed later in this report, this has implications for *in vitro* analysis. Although distinct in their embryology and physiology, the shared microenvironment of the thyrocyte and C cell are a further important consideration when examining tumour predisposition, cancer development and spread.

Elsewhere in the body, neural crest derived tissue forms a wide variety of functional tissues and structures. These include the peripheral nervous system, melanocytes within skin and elements of the autonomic nervous system (Adams and Bronner-Fraser, 2009). With regard to MTC, there are important associations including de-differentiation into neuroendocrine tissues such as Chief cells of the adrenal medulla from which pheochromocytoma arises and neuroendocrine elements in the gastrointestinal tract. Abnormalities of these tissues are features of multiple endocrine neoplasia (MEN2) and further discussed in section 1.3 (Raue and Frank-Raue, 2010).

1.2 Thyroid cancer

1.2.1 Thyroid cancer is the most common endocrine malignancy, with increasing incidence

Thyroid cancer is the most common malignancy of endocrine tissue, and can be classified into four main morphological types, papillary, follicular, medullary and anaplastic (undifferentiated) thyroid cancer (Cooper et al., 2009). Lymphoma can also arise primarily within the thyroid gland and the thyroid gland can be the site of secondary metastasis but neither of these two clinical entities will be considered further here. Papillary and follicular thyroid cancer, also known as well differentiated thyroid cancer, arise from follicular cells within the thyroid gland and, as previously discussed, function around follicular subunits to organify iodine and produce and secrete T3 and T4. The unique properties of thyrocytes in relation to iodine uptake provide specific targets for treatment in the form of radioiodine (^{131}I) which in part explains why the prognosis for these malignancies is much better than for MTC or anaplastic thyroid cancer (Tomoda et al., 2006).

The incidence of well-differentiated thyroid cancer is increasing and in particular the incidence of early well differentiated papillary thyroid cancer (Cooper et al., 2009)(Figure 6). Indeed, papillary thyroid cancer now makes up the vast majority of malignancies identified in most large series with a shift in rates compared to other types of thyroid cancer (Enewold et al., 2014). Much of the reported increase can be attributed from a greater detection of

early disease, much of which may be indolent in nature (Malandrino et al., 2013). A rising incidence in the diagnosis of thyroid cancer through more rigorous investigation of nodular diagnosis however is not leading to an increase in thyroid cancer mortality. Unlike PTC, MTC does not have an indolent form and no greater incidence of MTC has been reported.

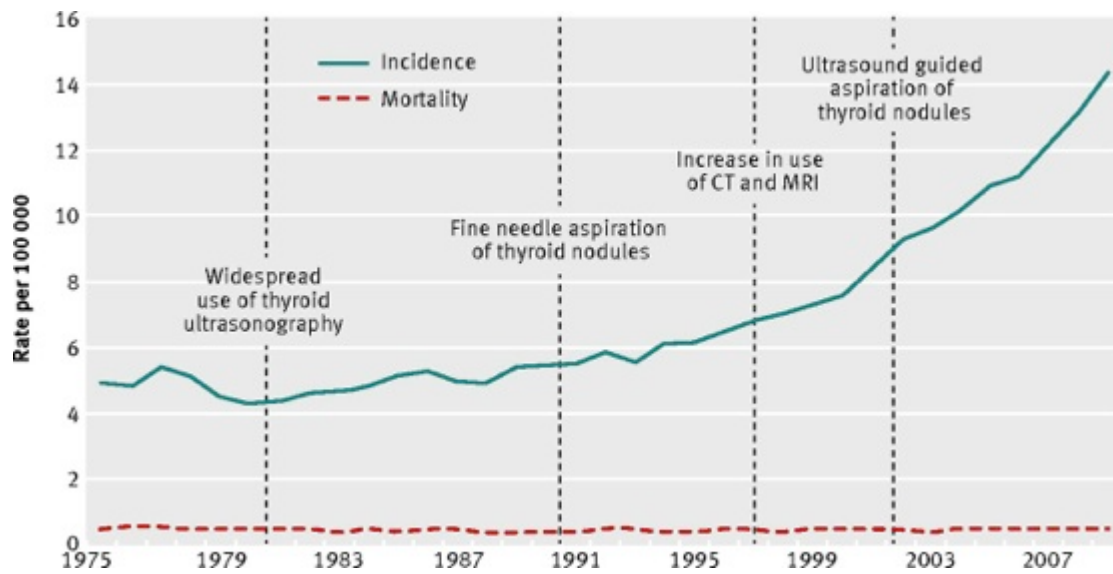


Figure 6. Incidence of and mortality from thyroid cancer in the US, 1975-2009 and advent of new technologies. (Brito, Morris and Montori, 2013).

MTC accounts for approximately 5% of thyroid cancers in the UK and US (Kloos et al., 2009). In 2008, examination of national data on all types of thyroid cancer revealed 2154 new diagnoses with a incidence in the UK of around 4.8 per 100,000 in women and 1.9 per 100,000 in men (CRUK, 2013). Although the incidence is increasing, this is, for the majority, made of an increase in the diagnosis of papillary thyroid cancer.

The ability to identify cancer at an earlier stage clearly has implications for patients with MTC and prognosis is linked to stage at presentation (Modigliani et al., 1998). Interestingly, at UHB, we have not seen locally the international increase in disease detection of early disease that has been reported (Smith

et al., 2014)(Brito, Morris and Montori, 2013). As discussed, the outcome in patients with MTC is linked to stage of disease at presentation. The fact that an increase in early disease identification is not seen locally seen has significant implications for patients with MTC as outcomes based on current trends and available treatments is unlikely to improve detection and therefore survival. There is however, no evidence internationally that MTC is being diagnosed at an earlier stage.

This increased detection of thyroid cancer comes both through a greater awareness of nodular thyroid disease and an increase in the use of imaging modalities such as ultrasound scanning and cross sectional imaging in the form of CT and MRI scanning (Brito, Morris and Montori, 2013). In spite of an increasing incidence, the mortality rate from well-differentiated thyroid cancer has remained stable, adding weight to the theory of early detection of indolent disease (lead time bias). National data from the cancer registry on the incidence and disease specific mortality in thyroid cancer highlights these trends (Cancer Research UK, 2013). Whether ultimately we are witnessing a lag in the mortality data and mortality will increase in line with incidence has yet to be seen. In contrast to well-differentiated thyroid cancer the incidence and rate of MTC has remained stable. This may reflect differences between these two types of thyroid cancer in that although micro-carcinomas occur in both, medullary micro carcinomas are progressive rather than indolent in nature.

1.2.2 Like other forms of thyroid cancer MTC most commonly presents with a neck mass in a clinically euthyroid patient

Where MTC presents primarily without a known familial predisposition (sporadic disease), its presentation is often indistinguishable from other forms of well-differentiated thyroid cancer. Most commonly, MTC presents with a neck mass, either within the thyroid gland or as locoregional neck nodal metastasis commonly in level six or in the lateral neck (Kloos et al., 2009). Presentation with locally invasive or distant disease occurs but is less common. Where MTC presents as part of a multiple endocrine neoplasia syndrome however, systemic symptoms secondary to phaeochromocytoma including diarrhoea and flushing, with signs of hypertension may occur prior to thyroid specific changes.

1.2.3 Investigation of MTC

As with other forms of suspected thyroid cancer, fine needle aspiration cytology (FNAC) in conjunction with a specialist performed ultrasound scan of the thyroid and neck are the mandatory initial assessment criteria (Perros, Boelaert, Colley, Evans, Rhodri M. Evans, et al., 2014). Most thyroid nodules that are investigated in clinical practice will be benign and as the incidence of MTC in a routinely investigated nodule is so low (0.83%), routine screening of all nodules with calcitonin estimation is unwarranted (Daniels, 2011). Where FNAC and USS characteristics are suspicious of MTC however, calcitonin estimation can be a useful adjunct to diagnostic work-up and is advocated in treatment planning by the American Thyroid Association (ATA) (Kloos et al., 2009). In many cases, where disease is limited to the thyroid gland, FNA and

USS will be insufficient to reach diagnosis of MTC and diagnostic thyroid lobectomy will be indicated (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014a). Where a diagnosis of MTC has been established prior to surgery, complete staging of both primary nodal and metastatic disease should be completed and will likely involve cross-sectional imaging of the neck, chest and liver to screen for secondary deposits as well as imaging of the adrenals to screen for phaeochromocytoma which may indicate a diagnosis MEN 2 and require treatment prior to the thyroid (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014c). Once a diagnosis of MTC has been established, conformation of RET (RE-arranged during Transfection) mutational status with genetic counselling and cascade testing as appropriate will be carried out and is a major tool for the identification of preclinical disease (Romei et al., 2011). Current recommendations for the RET mutation analysis include direct sequencing of germline DNA derived from leukocytes taken from peripheral blood with screening of exons in which RET mutations most commonly occur (exons 10, 11 and 13-16) (Eng, 1999). The exact exons tested and the algorithm for testing differs between laboratories.

1.3 MTC and multiple endocrine neoplasia type 2 (MEN 2)

MTC either occurs in isolation (sporadic MTC), in a familial form (FMTC) or in conjunction with other tumours of neuroendocrine origin (neural crest derived), multiple endocrine neoplasia (MEN) type 2A or MEN 2B (Kloos et al., 2009). MTC was first described as a malignant tumour with amyloid in 1906 but not histologically characterised until 1959 (HAZARD, HAWK and CRILE, 1959). Familial disease accounts for around 25% of cases of MTC with sporadic MTC accounting for the majority (75-80%). Since the advent of

diagnostic RET testing, identification of extrathyroid manifestations to distinguish FMTC from MEN 2A has become less important and the two entities are considered part of a spectrum of the same disease (Eng et al., 1996). Where extra thyroidal features are present, there is a variable presentation with phaeochromocytoma (approximately 50%) and primary hyperparathyroidism (30%) (Iihara et al., 1997). In MEN 2B there are additional clinical factors relating to abnormalities of neural crest derived tissue including Marfanoid facial features (high arched palate, mucosal abnormalities), amyloidosis and multiple cutaneous and gastrointestinal neuromas (Mulligan et al., 1993), (Donis-Keller et al., 1993), (Eng et al., 1995). MEN2 is caused by missense mutations of the RET proto oncogene with direct correlation between mutation and disease phenotype (Zbuk and Eng, 2007), (Eng et al., 1996). RET mutation-mapping forms the basis of clinical testing in MTC and MEN and forms the basis for cascade testing and clinical planning. In familial disease, RET mutations are causative in 85% of FMTC, 95% of MEN 2A and 98% of cases of MEN2B.

The study of these familial forms of cancer not only informs management of the patients and their families but it also provides important clues to the pathogenesis of the more common sporadic forms of the tumour. Thus, somatic mutation of RET has been detected in up to 60% of sporadic MTC (Eng and Mulligan, 1997). In addition, approximately 10% of patients with apparently sporadic MTC have a germline RET mutation and diagnostic testing, with subsequent cascade testing where appropriate, should be offered to all such patients (Eng et al., 1995) (Wohllk et al., 1996), (Schuffenecker et

al., 1997). This may reflect incomplete penetrance for MTC associated with a germline RET mutation although the *de novo* RET mutation rate has been estimated to be as high as 10% (Schuffenecker et al., 1997).

1.4 The molecular basis of MTC

1.4.1 The RET proto-oncogene

RET was identified in studies of NIH 3T3 cells transfected with lymphoma DNA as a novel rearranged protein; the coding region of which has retained the nomenclature (Takahashi, Ritz and Cooper, 1985). The RET gene encodes a receptor tyrosine kinase and, upon ligand activation by glial cell derived neurotrophic factor (GDNF) or one of its superfamily (GDNF family ligands (GLFs)), leads to RET receptor dimerization and activation (Jing et al., 1996). The RET gene is located on chromosome 10q11.2 and consists of 21 coding exons which, when transcribed, combine to encode a protein with three distinct domains (Takahashi and Cooper, 1987). The extra cellular binding domain contains 4 cadherin-like repeats in addition to a cysteine rich domain that is highly conserved across a range of species and in which many of the pathological mutations leading to MEN2 and FMTC occur (Figure 8 page 26) (Ponder, 1999). In addition, the RET tyrosine kinase has a hydrophobic, transmembrane domain as well as a cytoplasmic portion (Iwamoto et al., 1993). As a tyrosine kinase, RET is part of the superfamily of cell membrane bound growth factors that act as growth hormones and, when activated, lead to down-stream transcription and ultimately to cellular

differentiation and proliferation. As with other tyrosine kinases, the RET tyrosine kinase plays a key role in oncogenesis and plays a role in multiple cancers including thyroid, breast and lung (Gschwind, Fischer and Ullrich, 2004) (Mulligan, 2014).

Activation of RET with phosphorylation of the tyrosine residue tyr1062 causes downstream binding of SHC (Src homology 2 domain containing) producing a cascade of transcriptional activation which subsequently leads to cellular proliferation (Figure 7) (Zbuk and Eng, 2007). Mutations of the extracellular cysteine rich domain account for the majority of MEN 2A with C634W accounting for 85% alone (Zbuk and Eng, 2007), (Ponder, 1999). Cysteine codon mutations produce a RET receptor that is unable to form a disulphide bond with its intracellular binding partner (GFL-GFR α) and instead binds to another mutated receptor mimicking ligand binding with consequent up regulation. The missense mutation M918T (exon 16) gives rise to the majority of MEN2B with a mutation on the intracellular phosphorylation site and SHC binding domain. M918T is thought to increase RET-ATP binding affinity making RET more active (Wagner et al., 2012). It is rare to have a situation where familial cancer predisposition is caused by an activating mutation, where mutations of tumour suppressor genes are more commonly described (Chau and Wang, 2003) .

1.4.2 RET mutations in MTC

Activating missense mutations affect RET functioning in differing ways. The clinical presentation of the disease is phenotypically closely linked to the

position and effect of the point mutations in RET and as such RET phenotypes can be defined in broad terms at a molecular level (Zbuk and Eng, 2007). In FMTC, MTC arises in isolation and the majority of cases have a RET mutation (85%)(Eng et al., 1996). Where sporadic MTC arises with other neural crest tissue tumours the clinical syndrome is defined as multiple endocrine neoplasia (MEN) type 2A (pheochromocytoma and parathyroid hyperplasia) or 2B (pheochromocytoma, parathyroid hyperplasia and GI and cutaneous neurogangliomas) and almost always arise from a gain of function of the RET tyrosine kinase.(Donis-Keller et al., 1993) (Mulligan et al., 1993) (Eng et al., 1995). More than 98% of MEN2B has an identifiable RET mutation (Eng et al., 1996). Loss of function mutations of the RET tyrosine kinase predisposes to Hirschsprung's disease (abnormal development of intestinal neuronal ganglions) (Zbuk and Eng, 2007).

With the routine availability of diagnostic RET testing it has become apparent that there are families and individuals with a suggestion of a genetic predisposition to the development of MTC or CCH, for example with a young age at onset or a positive family history, in whom no RET mutation can be detected. Although possible explanations may include the presence of a RET mutation which has eluded detection or a sporadic occurrence of disease, it is likely that there are genes other than RET, predisposing to MTC/CCH which remain to be detected. The BTA has specifically recommended investigation of familial non-RET families in order to identify novel predisposing genes (Perros, 2007).

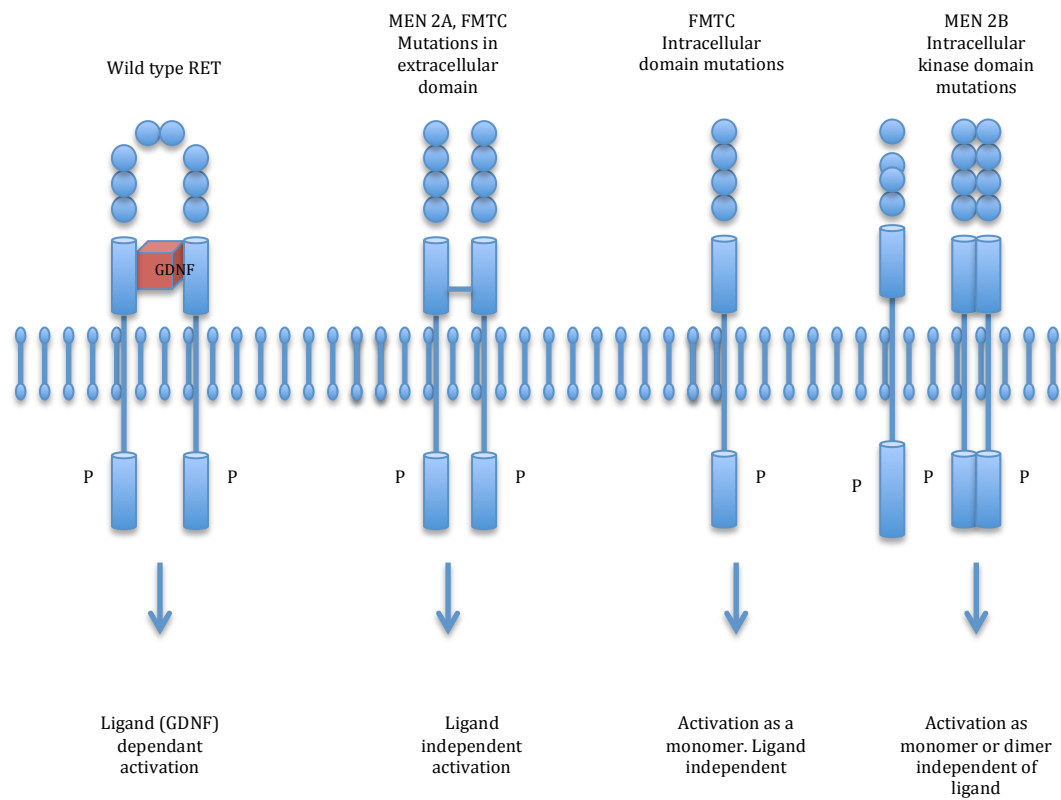


Figure 7. Activation mechanisms of the RET tyrosine kinase protein. Adapted from Mulligan (Mulligan, 2014).

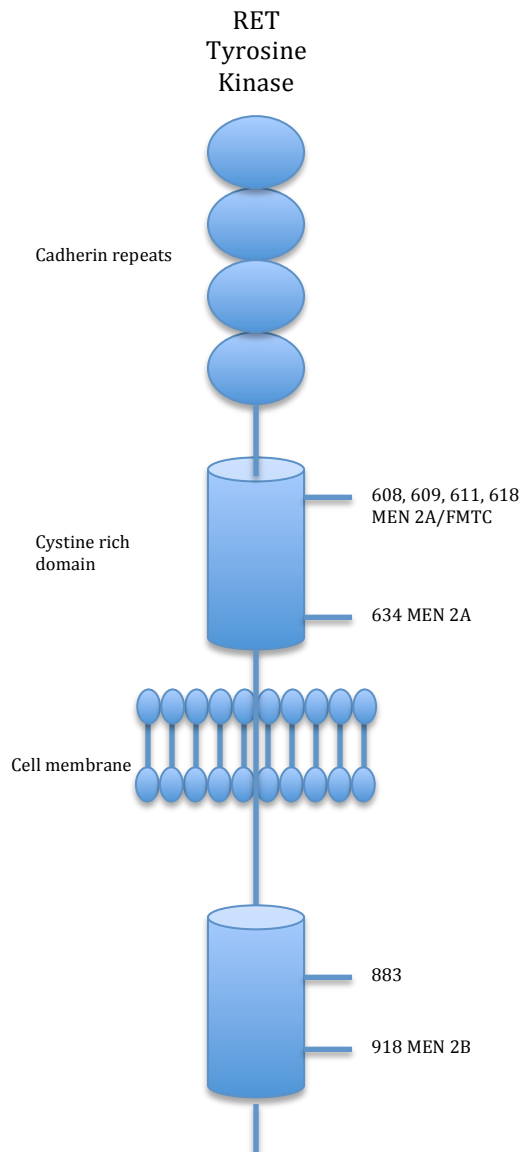


Figure 8. The RET (Re arranged during Transfection) proto-oncogene pathway and its downstream transcriptional activity (Zbuk and Eng, 2007).

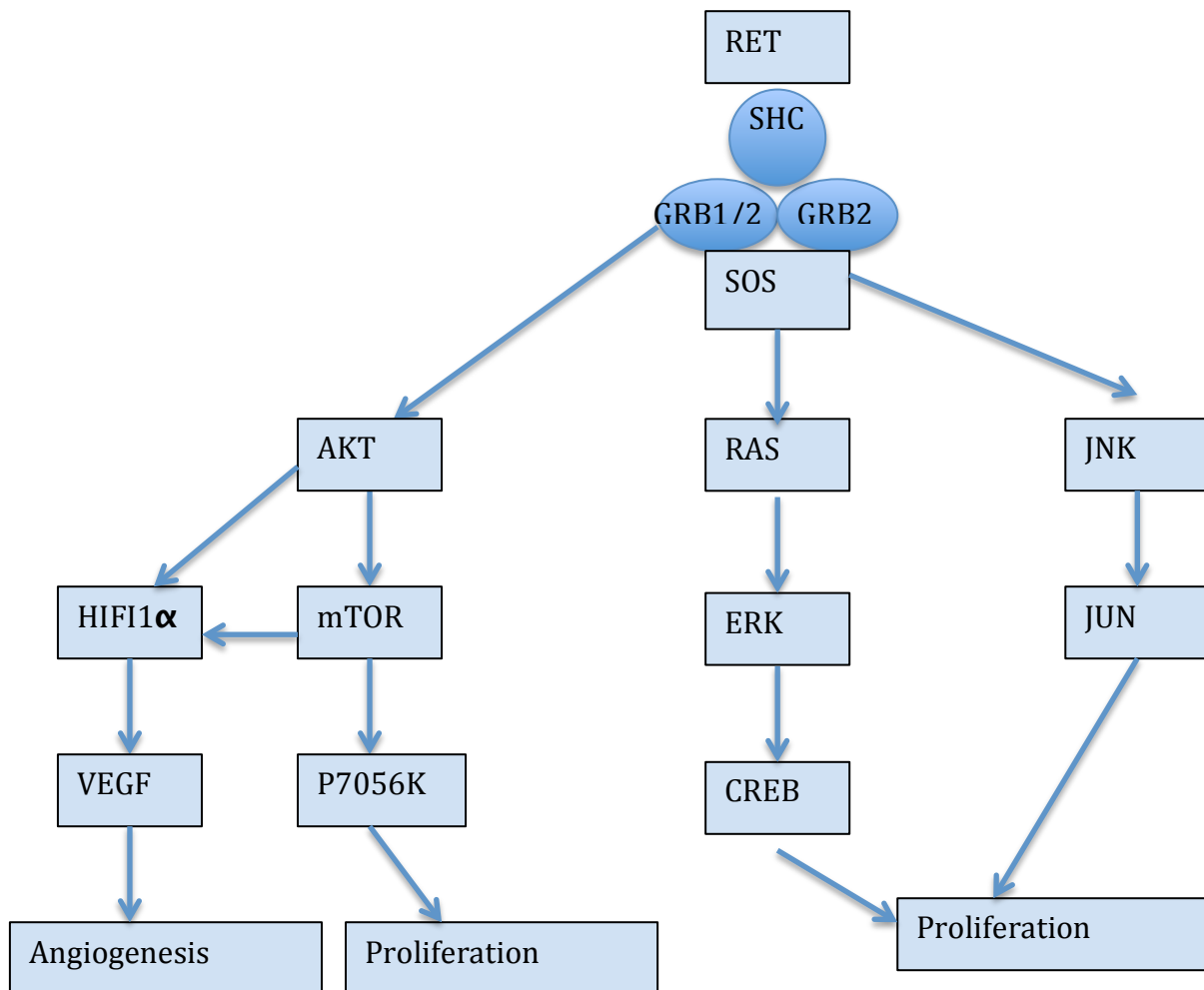


Figure 9. RET downstream stimulatory activation leading to cellular proliferation and angiogenesis via ERK, JKN, mTOR and VEGF pathways (adapted from (Mulligan, 2014)).

In MEN2, MTC presents either in isolation or with other tumours of neural crest origin, most commonly pheochromocytoma. Additional neural crest tumours are rare in sporadic (non-familial, RET negative) disease (Eng and Mulligan, 1997)(Ponder, 1999). MEN2 is regarded as a paragon of phenomics, where specific point mutations in the RET gene give differing clinical presentations of the disease and there is close genotype phenotype correlation (Zbuk and Eng, 2007). It appears for example that mutations affecting the intracellular domain (most commonly M918T) of the transcribed transmembrane tyrosine kinase, lead to more severe forms of the disease, typically MEN2B, with

younger age at disease onset and more aggressive clinical course. In contrast, activating missense mutations leading to RET tyrosine kinase structural anomalies in the extracellular domain (most commonly p. C643W) lead to milder forms of the disease, with the distance from the membrane being the most important factor in disease severity (Zbuk and Eng, 2007). Mutations at maximum distance from the cellular membrane typically cause FMTC that tends to present later and has rare association with other neural crest tumours. This may have important implications within the context of this study with regard to the likely genotype aberrations leading to the identified phenotype within our index family.

1.5 The treatment of MTC

1.5.1 Non-surgical treatment of MTC

MTC is a surgical disease, with complete resection representing the only chance of permanent cure (Kloos et al., 2009). Surgery is also indicated for the prophylactic management of RET mutation positive familial disease and for local control of symptomatic but incurable local disease. As C cells do not possess the ability to uptake iodine, radioiodine therapy is not effective and goes some way to explaining the disparity between survival outcomes in MTC versus well differentiated thyroid cancer (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014c). In addition, MTC is relatively refractory to external beam radiation and its use in primary treatment and treatment of recurrent disease is controversial (Schwartz et al., 2008) (Brierley et al., 1996). Standard chemotherapeutic agents are not recommended in MTC due

to lack of tumour response (Kloos et al., 2009). Small molecule inhibitors of tyrosine kinase receptors such as Vandetanib have shown some promise in inducing tumour remission in more recent trials. However, none have been shown so far to improve life expectancy (Wells et al., 2012). This highlights the need for better understanding of the genetic events leading to MTC as a better understanding of the downstream effector pathways is likely to reveal further molecular targets. As a surgical disease with limited adjuvant treatment options, survival and prognosis are very much linked to the stage of disease at presentation. As such, survival in stage one disease at 10 years is as high as 100%. MTC often presents late, however, with 50% of patients at stage III or stage IV at diagnosis with five-year survival in advanced (stage IV) disease is only 21% (Roman, Lin and Sosa, 2006).

1.5.2 Who should manage patients with MTC?

There is a move towards centralisation of cancer services in the UK and internationally and much debate regarding how best to manage patients with rare malignant diseases. MTC tends to be managed in the UK in tertiary centres, although significant numbers of patients are treated outwith of large centres. As regional genetics services generally coalesce in tertiary centres, there is certainly a logical argument based on geographical placement of services for this taking place. Recent debate on thyroid surgery has focused on the nature and training of surgeons undertaking thyroid surgery, of which the most important factor is the volume of surgery being undertaken by individuals and individual centres (Oltmann and Holt, 2014). It is clear that where high volumes of surgery are performed the outcomes are better and

there are consensus statements from national frameworks in support of this (BAETS national audit (Anon, n.d.)). Although initial surgery in MTC is often routine and performed without the knowledge of the diagnosis, subsequent surgery and further surgery for recurrent disease in the neck is challenging and should only be undertaken in large centres. I reviewed UK and US perspectives on these issues to provide an editorial with recommendation based on the on current evidence (Smith, Watkinson and Shaha, 2012). As the surgical treatment of MTC often involves dissection of the central and lateral nodal compartments of the neck as well as the surgical management of recurrent disease, regional concentration of cases to specific units is even more important for patients to receive optimal treatment.

1.6 Cancer as a genetic disease

Mutations in genes that ultimately lead to alterations in the structure and therefore function of cell cycle regulator proteins are features of most cancers and vital for the confirmation of cellular reproductive function leading to clonal expansion of mutated cell lines. On a fundamental level, cancer is a disease of genes and gene regulation and most cancers are polygenetic (Vogelstein and Kinzler, 2004). Inherited mutations therefore only confer a propensity to develop cancer and many of the mutations in cell cycle regulatory pathways that eventually develop are somatically derived and may be secondary to environmental factors (Stratton, Campbell and Futreal, 2009). In an ideal state, tissues exist without growth or development, but in quiescence with loss and natural replacement of cells as required (Loeb and Harris, 2008). The

reality is somewhat different however, with constant changes in the genetic architecture often brought about through environment factors that cause direct DNA damage such as free radicals, ionising radiation, carcinogens and viruses (Talbot and Crawford, 2004) Although DNA replication has a very high fidelity, errors develop, a process accelerated by exogenous mechanisms of DNA damage (Lengauer, Kinzler and Vogelstein, 1998). It is the combination of inherited phenotype and genomic instability that ultimately pushes the balance within tissue in favour of malignancy.

Groups of proteins that, when abnormal, can predispose to cancer include growth hormones and their receptors, intracellular transducers and receptors, DNA repair and cell cycle control proteins and proteins that induce apoptosis (See Figure 10)(Lodish et al., 2000). Somatic mutations that may lead to cancer include single point mutations (substitutions, insertions and deletions), translocations, whole chromosomal amplifications and copy number increases and decreases (Stratton, Campbell and Futreal, 2009). As the methods employed in this project using whole exome sequencing (WES) in the identification of novel predisposition gene mutations will generate a significant quantity of non-relevant data, using 'biologically relevant pathways' as a method of data filtration is a well recognised tool and forms part of the rationale for targeted focus on specific mutations.

1.6.1 Oncogenes and proto-oncogenes

Oncogenes are genes encoding for proteins that transform a cell or tissue in culture or in an animal to cancer. Few of these are derived from proto-

oncogenes that are normally occurring genes encoding proteins involved in cell cycle regulation (Pillai, 1992). To transform a proto-oncogene into an oncogene producing an onco-protein usually involves a gain in function, as highlighted by the gain in function mutation of the RET gene via well described missense mutations of the tyrosine kinase domains. Gain of function mutations account for the majority (90%) of disease causing somatic mutations (Mulligan, 2014). Other gain of function mutations include those in proto-oncogenes such as RAS and BRAF, with p. V600E BRAF mutations being the most commonly described activating mutation in human cancer (Vogelstein and Kinzler, 2004). Other than point mutations, translocations such as RET/PTC rearrangements where a regulatory gene is inappropriately brought under the promoter region of another gene, and gene amplifications where there is localised over amplification of regional DNA sequence, may lead to inappropriate downstream gene amplification. Although in the example of point mutations, an oncoprotein is produced that has as a potential differential function to the original protein, in the examples of reduplication and translocation, abnormal amounts of a morphologically normal protein are produced (Lodish et al., 2000). This is an important consideration within the context of this study owing to the pathway up-regulation hypothesis proposed in Chapter 5.

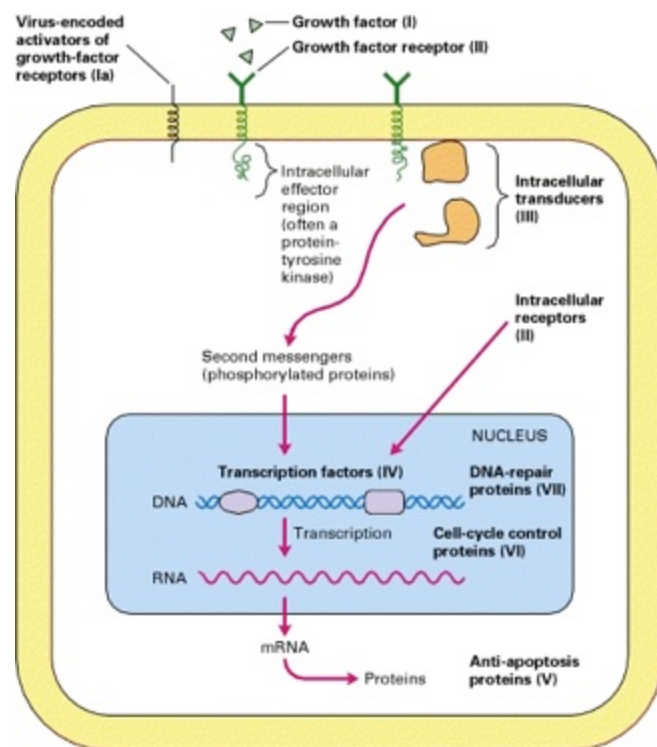


Figure 10. Schematic representation of proteins and pathways in which mutations and functional changes can lead to cancer predisposition (Lodish et al., 2000).

In well-differentiated thyroid cancer, RET/PTC rearrangements provide evidence of up-regulation of constitutively normal RET tyrosine kinase can cause cancer. Common abnormalities in well differentiated thyroid cancer include point mutations in the BRAF and RAS pathways and also the RET/PTC and *PAX8/PPARG* rearrangements with point mutation BRAF V600E being the most common (Leeman-Neill et al., 2013). There are a number of RET/PTC rearrangements than effectively bring RET under the promoter region of other genes. RET/PTC rearrangements are more common in patients with thyroid cancer previously exposed to radioiodine where high levels of ^{131}I , concentrated by the follicular cells have DNA damage induced by direct gamma radiation (Leeman-Neill et al., 2013). In these circumstances

RET is constitutively normal but direct over expression leads to activity similar to that of an oncoprotein.

1.6.2 Tumour suppressor genes

The protein products of tumour suppressor genes are cell cycle inhibitors that provide a balance between cellular proliferation and cell death by direct inhibition of the cell cycle with promotion of apoptosis (Chau and Wang, 2003). Furthermore, tumour suppressors play a role in DNA repair and as inhibitors of cellular migration (Friend et al., 1986). Although tumour suppressor genes undergo a range of mutations typical of all genes, mutations that lead to loss of function of a tumour suppressor are classically described as recessive in nature and usually caused by point mutations or small insertions or deletions. The identification of the recessive nature of tumour suppressor gene inheritance lead to development of the two-hit hypothesis initially proposed by Knudson in 1976, and still widely employed in tumour suppressor gene research (Knudson et al., 1976). In his studies of patients with retinoblastoma (RB), Knudson recognised that a predisposition to the disease could skip generations within families, with others arising sporadically, without a family history (Knudson, 2002). Time to development in tumours occurring in patients with a family history was much sooner than those without and the rate of bilateral disease much higher in hereditary disease. Assuming that both RB1 genes needed to contain an error to lead to disease, by mathematically modelling rates of disease between two groups it could be predicted that time to disease development in those with a single gene defect (needing a single mutation in the unaffected allele) to be

significantly different to those without a gene defect requiring mutations of both alleles (two-hits) to develop disease.

More recently some modifications to this hypothesis have been described which move away from the classic two point mutational theories. Haploinsufficiency; where a mutation of a single allele leads to an incompletely functional gene, which may rather than being absolute, be a product of the tissue in which the gene product is expressed or changes in the microenvironment (Payne and Kemp, 2005). As the rate of spontaneous mutation is so low, evolutionary theories of cancer development suggest the rates of cancer seen far outstrip that that would be expected by the two hit hypothesis alone. This adds weight to the theory that haploinsufficiency may confer a selective advantage within tissues, the so called 'mutator' phenotype (Loeb, 1991). Further, mutations in tumour suppressors can confer a gain of function or provide a dominant negative effect.

1.6.3 Mutations that lead to cancer: Natural selection in cancer tissues

Neoplasm evolution by mutation of normal cells within tissues gaining a selective advantage has been proposed as the model of cancer development since the mid 1970s (Nowell, 1976). Cancer is a disease of genes, where gene mutation provides opportunity for cellular progression, clonal expansion, evolution and competition with cells in surrounding tissues (Greaves and Maley, 2012). Macroscopically, cancers contain multiple cell populations with differing mutation profiles leading to a heterogeneous mix within a tumour one often finds in pathological specimens (Cairns, 1975). One major limitation of histopathological examination is that specific cell properties cannot be

predicted by cell appearance and the cell characteristics are driven by mutation profile rather than outward appearance. Neoplastic heterogeneity is driven by somatic mutations that give individual cells specific properties and form complex mosaics competing for resources (Merlo et al., 2006). In this way, on a microscopic scale, tumour evolution mirrors the evolutionary pathways and pressures experienced by other species (Merlo et al., 2006).

Tumour cells are more genetically unstable than normal tissues and more likely to develop further mutations (Merlo et al., 2006). Thus, within tissues, a single initial mutation may predispose cells to development of further mutations and as such set up a somatic evolutionary pathway (Gerlinger et al., 2012). This initial mutation may be tissue specific as a mutation in a gene may give selective advantage in one microenvironment but not in another (Gerlinger et al., 2012). Once mutated, populations of cells within malignant tissue may live and develop symbiotic relations with cells with differing characteristics, for example, cells have the ability to invade blood vessels, evade the immune system whilst others have the ability to migrate through blood and lymphatics. Significant intra-tumoural evolution has been demonstrated with further unique characteristics found in metastases and distant disease although solitary mutations are usually found within all samples (Merlo et al., 2006)(Gerlinger et al., 2012). Specific properties of a tumour, whether is indolent or aggressive, whether it is locally invasive or metastasises may be due to individual or dominant properties of the cell mix within a tumour. The differing cell types found in tumours may also to a greater or lesser extent, be competing for the resources supplied to the

surrounding microenvironment as well as those of normal tissue. The interplay between these factors and the host environment is likely to provide an explanation for the specific clinical presentations seen and in part explain why tumours of the same histological type behave in different ways.

It is clear that in heterogeneous tumours such as MTC, the cells within the tumour carry different characteristics to those of the original progenitor mutations that gave cells within a tissue a selective advantage. The most commonly found somatic mutation is of the RET gene, and it is likely that forms the driving mutation for most cases of MTC. Both C-cells and thyrocytes share a microenvironment within the thyroid and RET mutations are common to MTC and PTC through the RET/PTC rearrangement. This provides evidence that upregulation of the RET pathway is important in the microenvironment of both C cells and thyrocytes.

1.7 Hypothesis and Aims

MTC is a rare but aggressive disease where surgery offers the only chance of cure (Kloos et al., 2009). Surgery is only curative in early stage disease. The majority of patients with MTC present late, where the chance of long term cure is significantly lower. In familial disease, identification of disease causing mutations of the RET proto-oncogene allow prophylactic surgery to be offered to at risk individuals, and offers good rates of disease control (Kloos et al., 2009)(Eng et al., 1998). In families without RET mutations, and in the more common sporadic disease, no such tests exist. A better understanding of the

molecular genetic events in MTC is needed such that further genetic tests, biomarkers and targets for novel therapies can be developed. In depth analysis of rare cancer phenotypes have demonstrated high likelihood of identifying such events and the study of such phenotypes informs the management of more common, sporadic disease. The aim of this project is to identify and understand these events.

Aims

1. To assess familial non-RET MTC using next generation sequencing to identify potential disease predisposition loci.
2. To assess the presence of identified abnormalities in a wider cohort of sporadic disease
3. To assess the functional effects of identified abnormalities

Chapter 2.

Materials and Methods

2.1 Project design and recruitment

2.1.1 Ethical approval

Ethical approval for the project was sought and granted from the National Research Ethics Service and University of Birmingham:

Awarding body	Reference
National Research Ethics Service (NRES REC)	CA/5175
University of Birmingham (UoB) Ethics	ERN_11-0775

2.3.2 Recruitment through local physicians and surgeons and access to local databases

Recruitment for the project commenced in August 2010. The University Hospital Birmingham NHS Trust (UHB) is a tertiary referral centre for both Head and Neck and Thyroid cancer. Birmingham Women's Hospital (BWH) houses the regional genetics referral service and as such handles local, regional and in the case of some diseases, national referrals. To gain the maximum coverage for patient recruitment, a search strategy from both centres was employed. Initially, using the thyroid cancer database, a prospective database of all thyroid operations and malignancies maintained by the Head and Neck Department at UHB over the last 18 years, hospital records of all patients treated at UHB with a diagnosis of MTC were analysed. The database allows comprehensive analysis of details on all patients surgically treated at the UHB, as well as a portal of access for pathological

records, and clinical and follow up data. In addition to this, the clinical genetics files (CGU files) for patients referred for clinical testing with suspected or proven MTC either as an index case or as an individual with a positive or suspected family history were analysed. The current BTA guidelines recommend all patients with a diagnosis of MTC be referred for genetic testing and counselling and as such there was overlap between the two databases in terms of patients (Perros, 2007). There were, however, also differences with some patients receiving primary treatment outside of UHB prior to being referred for genetic counselling and testing. In addition, there was a cohort who had not undergone surgical treatment at UHB but were under the care of the genetics department at BWH.

Familial cases and those with young onset sporadic disease are most likely to harbour identifiable genetic alteration and as such were targeted first for recruitment (Table 1). Recruitment consists in order of preference:

Familial non-RET MTC
Young onset sporadic non RET MTC (Under 40 at diagnosis)
Other sporadic non-RET MTC
RET positive sporadic MTC
RET positive Familial MTC (MEN2A/2B)

Table 1. Recruitment strategy in order of preference

2.3.3 Recruitment through research meetings

Several meetings were targeted as likely to have significant numbers of clinicians with a special interest in MTC. Focus was placed on surgical meetings, as surgeons are most often the primary treating clinician, as well as the person most likely to follow the patient long term. The project was advertised as a poster presentation at the British Association of Thyroid and Endocrine Surgeons (BAETS) in Birmingham in October 2010 where flyers and business cards were also handed to delegates (Appendix 1). Further advertisement was performed at the BAETS meeting in Poitiers, France in Oct 2011 and in Cardiff, Wales in Oct 2012. Preliminary data from this project was presented orally at the BAETs in Rome 2013 (receiving the British Journal of Surgery (BJS) prize for best oral presentation) and several families were recruited as a direct result (see appendix 2). In addition, flyers and advertisement was performed at the BTA in Newcastle in 2011 and through the BTA newsletter. Furthermore, the preliminary data and research methodology has been presented orally both at the European Society of Endocrine Surgeons (ESES) in Cardiff in May 2014 and at the American Head and Neck Society (AHNS) in New York in July 2014.

2.3.4 Recruitment through direct physician contact

Through these meetings, collaborative links for recruitment were established with Great Ormond Street through Tom Kurzawinski, Consultant Endocrine and Paediatric Surgeon, the Freeman Hospital in Newcastle through Professor Tom Leonard, Consultant Endocrine Surgeon and the Glasgow General through Louise Clarke, Consultant ENT surgeon. In addition we have

established clinical collaborative contacts with Patrick Morrison in Northern Ireland and David Scott-Coombes, Consultant Endocrine Surgeon in Cardiff.

2.3.5 Recruitment through charitable organisations

Further advertisement was done via the internet through contacts at the AMEND charitable trust, the British Thyroid Foundation (BTF) and the Butterfly Cancer Trust who all posted information regarding project recruitment on their websites. In addition, the AMEND charitable trust, through contacts with their patient groups, hold a registry of patients with disorders of the neuroendocrine system. The BTA advertised the project through their newsletter Collaboration with their chief executive Jo Grey allowed communication through their organisation



Figure 11. Online advertisement of the project via collaboration with AMEND (2011 newsletter), and the British Thyroid Foundation website (newsletter 2011).

<http://www.btf-thyroid.org/index.php/resources/news-archive/medullary-thyroid-cancer-study>

http://www.google.co.uk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CC8QFjAA&url=http%3A%2F%2Fwww.amend.org.uk%2Fresources%2Fnewsletters%2Fdoc_download%2F95-q4-2011-

[newsletter.html&ei=llruUdLzDMWa1AWa7ICYDg&usq=AFQjCNGKzcWHbyddGOgfK_kb4IVKLe6xcQ&sig2=OgnyKltmTa7Q0Oh4YoRuDQ&bvm=bv.49478099.d.d2k](http://www.google.co.uk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CC8QFjAA&url=http%3A%2F%2Fwww.amend.org.uk%2Fresources%2Fnewsletters%2Fdoc_download%2F95-q4-2011-newsletter.html&ei=llruUdLzDMWa1AWa7ICYDg&usq=AFQjCNGKzcWHbyddGOgfK_kb4IVKLe6xcQ&sig2=OgnyKltmTa7Q0Oh4YoRuDQ&bvm=bv.49478099.d.d2k)

2.3.6 Recruitment through contact with other research groups

In addition to recruiting patients directly to the study, an attempt was made to access archived tissue through collaboration with other groups identified through research networks. Literature searches were performed to identify groups with recent publications consisting of cohort of patients with MTC in whom genetic and or functional research had been performed. The principal investigators of eight groups worldwide were contacted to request collaborative links and gain access to fresh and archived tissue. Through establishing links with Professor Stan Sidhu's group in Sydney and Professor Robledo's group in Madrid we were able to gain access to multiple samples of DNA extracted from peripheral blood (lymphocytes), fresh tissue and FFPE embedded tissue. This has included samples from both familial CCH/MTC and sporadic disease. This enabled us to expand the scope of the search for somatic mutations in MTC far beyond what would have been possible using local or even national recruitment and tissue examination.

2.3.7 Research protocols

Where appropriate patients were identified, they were contacted using standard introduction letters and invitations amalgamated together in a study pack. Consent for those patients interested in being in the study was obtained by EW (principal investigator) and a file created as part of the National Institute of Health Research (NIHR) held by Professor E Maher (ERM). Recruitment involved informed consent to participate, permission to access medical records for full details on disease presentation and management. Tissue blocks were requested from which to extract paraffin embedded,

formalin fixed DNA and a blood sample was obtained so that leucocyte, germline DNA can be extracted. Recruitment is on-going.

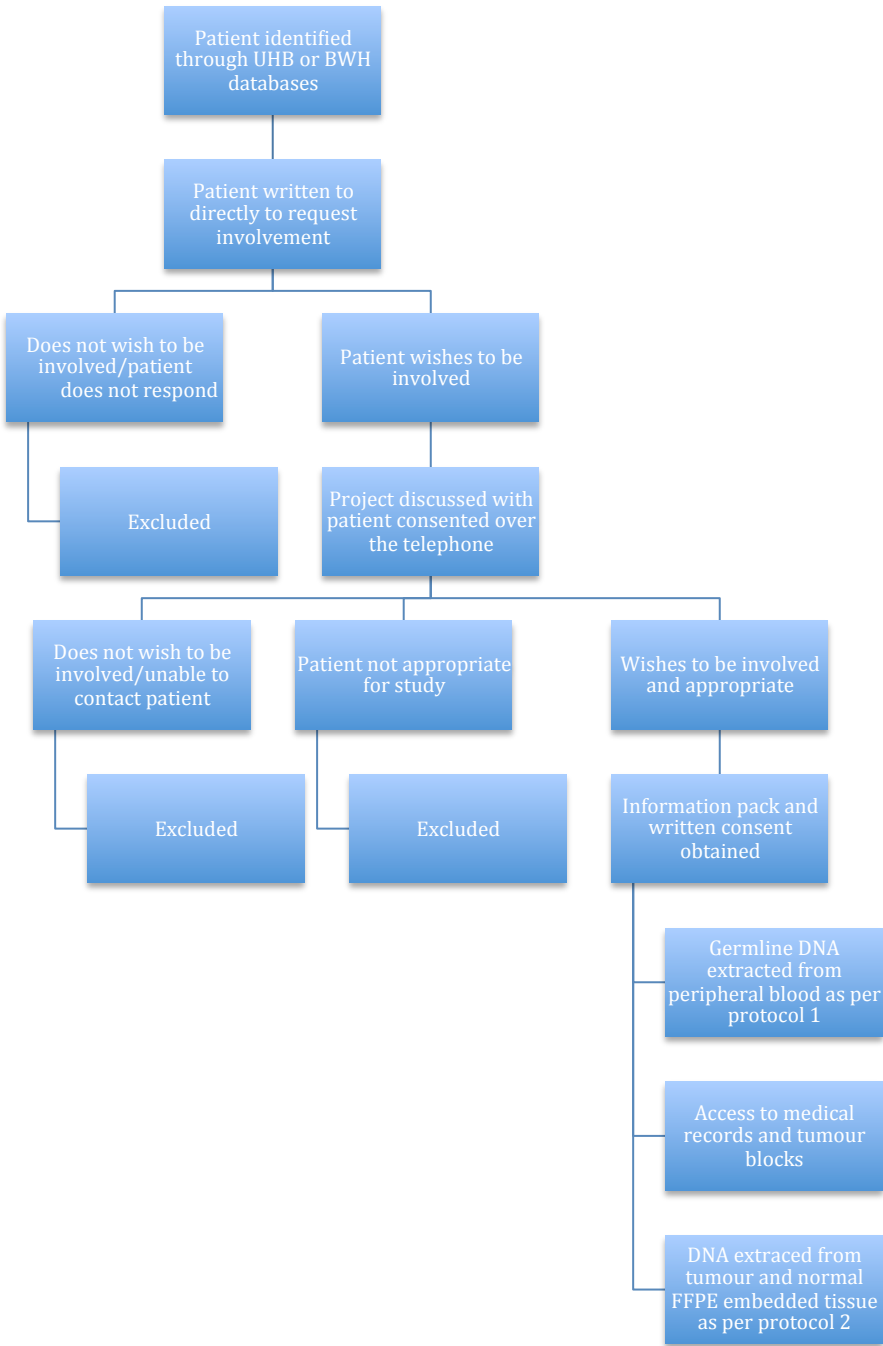


Figure 12. Protocol for enrolment of patients identified through UHB/BWH databases

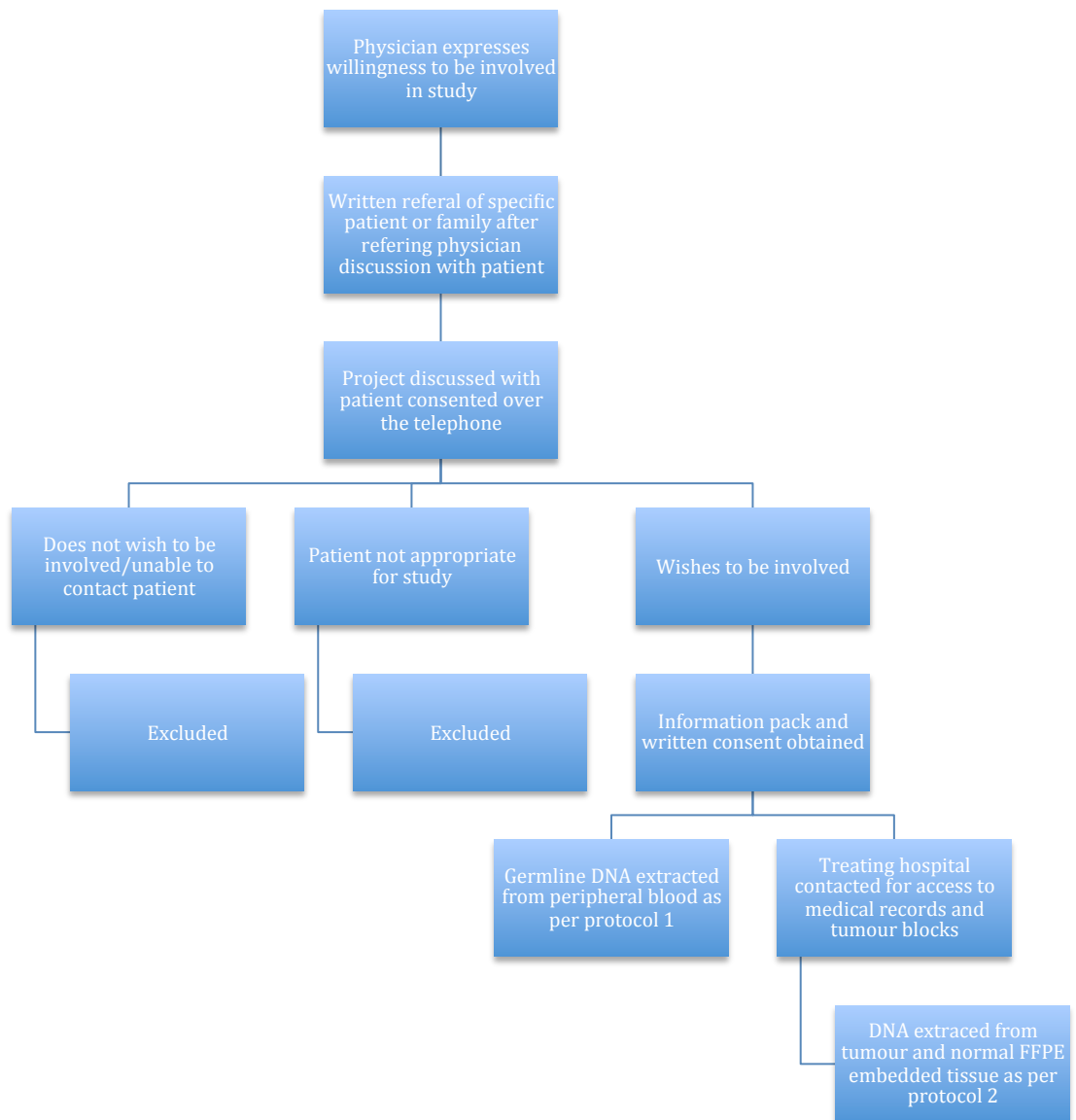


Figure 13. Protocol for enrolment of referred patients from other physicians

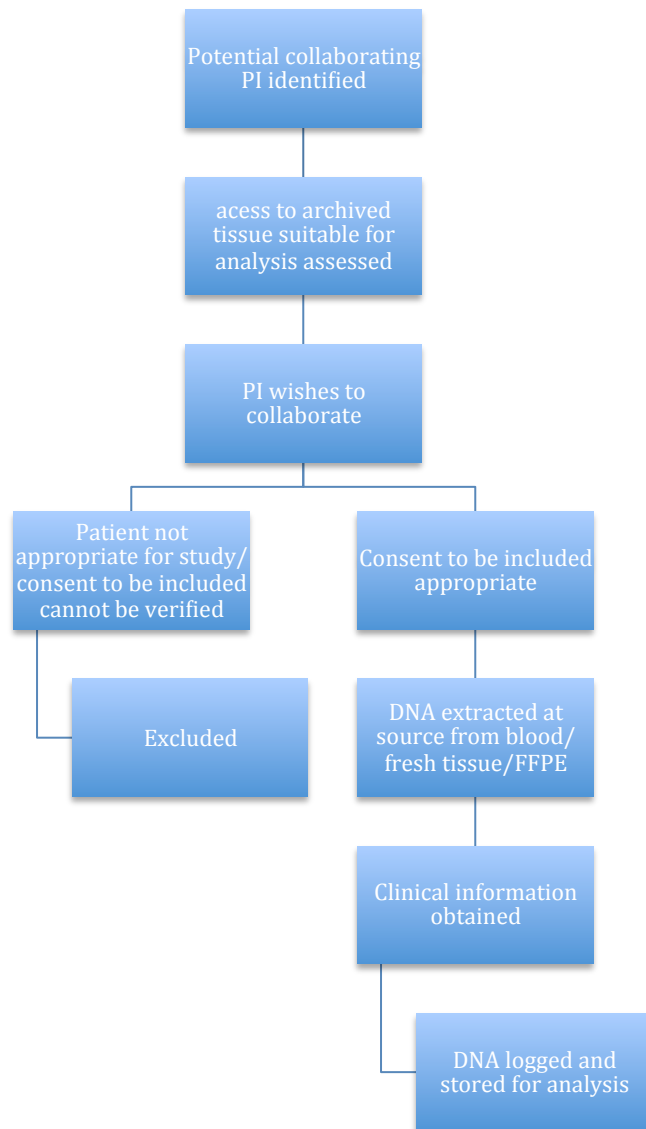


Figure 14. Protocol for contact and access to tissue and DNA held by collaborating groups

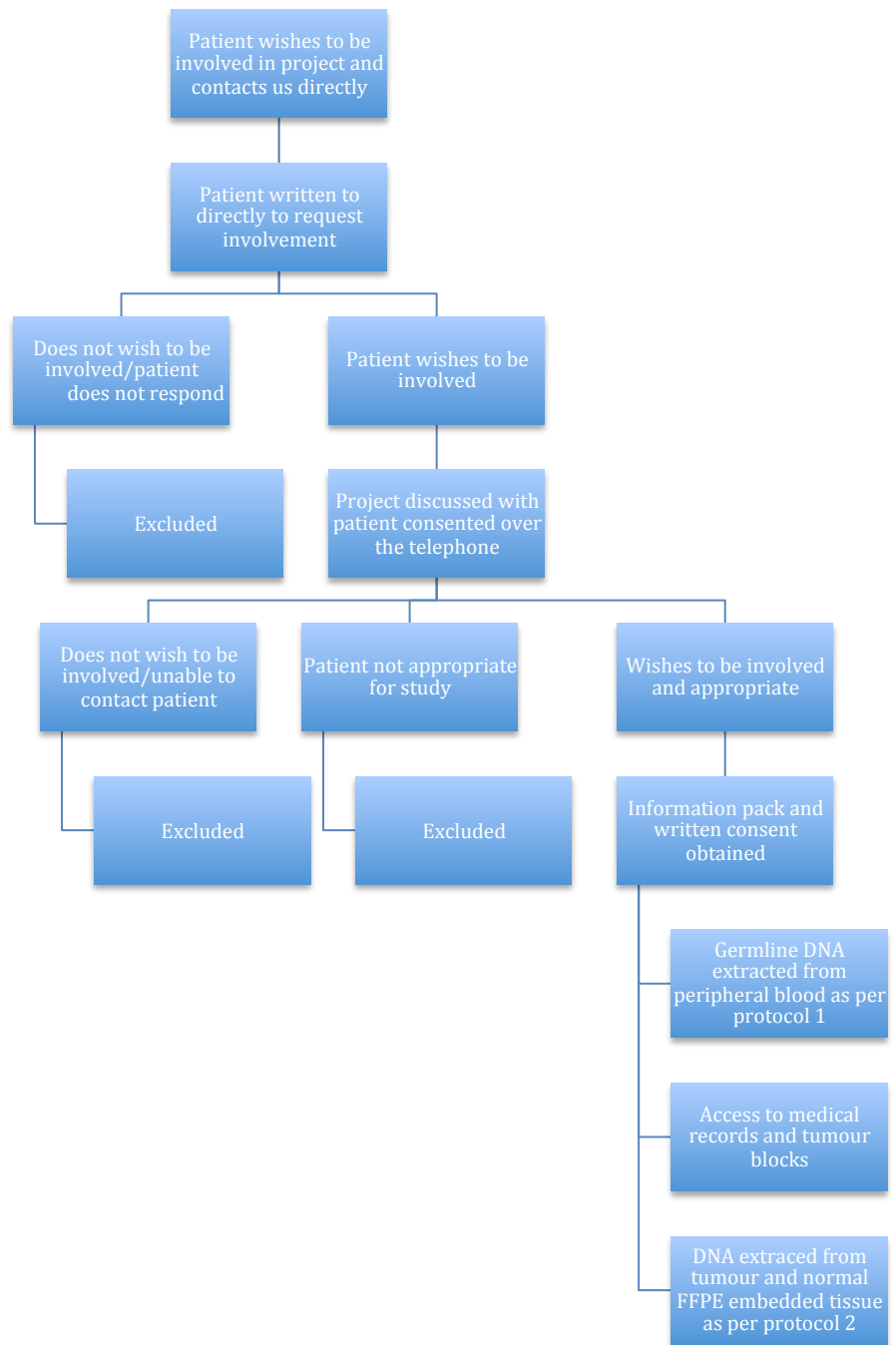


Figure 15. Protocol for recruiting patients contacted through patient lead research databases

2.2 Sequencing

All reagents were obtained from Sigma-Aldrich unless otherwise stated (Poole, UK)

2.2.1 Primer design and Polymerase Chain Reaction (PCR)

Primer pairs were designed using the online software, Primer 3: (WWW primer tool; University of Massachusetts Medical School, USA). University of California, Santa Cruz (UCSC, USA) databases were used to identify consensus sequences against which to design primers (Anon, n.d., p. 3). Primers were designed to bind to regions approximately 30-50 base pairs (bp) in the up or down stream pathways of the forward and reverse strand of each exon to minimise reading artefact around the exonic region.

Initial PCR was performed using Faststart Taq DNA polymerase chemistry as per manufacturer's instructions (Roche, Indianapolis, USA). Reagents were kept on ice and thawed prior to use and gently mixed by pipetting prior to PCR. Experiments were carried out using thin walled 96 well plates suitable for use within a thermal cycler (Tetrad 2, BIORAD, Gene Amp PCR system 9700). PCR conditions are shown in Table 2. Thermal cycling properties are shown in Table 3. Amplicons were run out on an agarose gel to verify PCR success and measure amplicon size prior to sequencing.

<i>PCR Reaction components</i>	<i>1 X volume (µl)</i>
10 X PCR buffer (20mM MgCl₂)	2.5
5x GC rich solution	2.5
2mM dNTP mix (1 to 1 ratio)	2.5
Primers (forward and reverse)	1.0
Taq Polymerase (Roche)	0.2
400ng template genomic DNA (leukocyte derived)	2.0 (after dilution if needed)
dH₂O	Made up to 25
Total	25

Table 2. Taq polymerase PCR conditions

<i>Step</i>	<i>Temperature</i>	<i>Duration (Min)</i>	<i>Cycles</i>
1. Hot Start	95 °C	5	-
2. Denaturing	95 °C	30-45s	
3. Annealing	58 °C	30-45s	
4. Extension	72 °C	Dependent on length of PCR product (Approx. 1kb/min)	
Cycle to step 2. 25 times			
5.Final Extension	72 °C	10	

Table 3. Thermal cycling properties (BIORAD Tetrad 2)

2.2.2 Agarose gels (PCR product estimation prior to DNA sequencing)

A 1.5% agarose gel (1.5g agarose) was made by mixing 1X TBE (Tris-borate-EDTA) made up to 200ml. The mixture was heated to boiling and swirled to mix the powder until completely dissolved. Once cooled, ethidium bromide was added to a final concentrate of 0.5 µg/ml. The gel was poured into a gel casting tray with the appropriate number of combs to run each reaction in a separate lane with a DNA quantification ladder in each comb. The gel was allowed to set completely at room temperature (RT) (approximately 20min). The combs were removed rubber ends if the casting tray were removed and the gel and tray place in an electrophoresis chamber containing enough

1XTBE to cover the gel and the wells. 5 µl of PCR complete DNA was loaded into each well with an equal v/v of loading dye (6X loading dye; New England Biolabs, Ipswich, USA). DNA quantification ladder was loaded to enable PCR band size recognition and the electrophoresis chamber run initially at 120mV increasing after 5 min to 180mv for a further 15min or until the gel and ladder had migrated sufficiently to allow quantification (1kb DNA ladder, New England Biolabs, Ipswich, USA).

2.2.3 Exosap reaction

The exosap reaction is a clean-up step required in sequencing to ensure clarity of the sequence and minimise signal noise created by unwanted products of the PCR reaction including unused primers and nucleotide triphosphates (dNTPs) that may affect sequencing.

<i>Exosap Reagent</i>	<i>Volume (µl)</i>
Fast AP (alkaline phosphatase) 1u/µl	1
10X FastAP buffer	1
Exonuclease I 20u/µl	0.25
PCR product	5
H ₂ O	2.75

Table 4. Exosap PCR clean-up conditions

The reaction components were mixed well then incubated at 37 °C for 30 min in a thermal cycler before deactivating the enzymatic component by increasing the temperature to 85 °C for 20 minutes.

200-500ng of purified PCR product was used in cycle sequencing reaction which involves the amplification of one strand of the PCR product using a

single primer. Dye terminator chemistry kits were used as per manufacturer's instructions (Big Dye Terminator cycle sequencing kit v3.1: Life technologies , Paisley, UK)

<i>Reaction components</i>	<i>1 X volume (µl)</i>
<i>5 x sequencing buffer for</i>	<i>2.0</i>
<i>Big Dye Terminator v3.1</i>	<i>0.75</i>
<i>Primer (forward or reverse)</i>	<i>1 (3.2pMol)</i>
<i>Purified DNA product</i>	<i>200-500ng</i>
<i>dH₂O</i>	<i>up to 10 µl</i>

Table 5. Sequencing PCR conditions using Big Dye terminator chemistry (Life Technologies).

	<i>Temperature (C)</i>	<i>Duration</i>
1. Denaturation	95	25s
2. Annealing	60	25s
3. Extension	50	3min
4. Cycle to step 1 X 28		
5. End		

Table 6. Thermal cycling conditions for sequencing PCR

2.2.4 DNA precipitation

3.5 µl of precipitation buffer (0.5M EDTA equal v/v with sodium acetate (NaAc)) was added directly to the 96 well plate post sequencing PCR product volume of with 100 µl of 100% ethanol. A pellet was formed by centrifugation in a 96 well plate centrifuge at 4000rpm for 13minutes at 4 °C. The plate lid was removed and the plate centrifuged at 400rpm for 1 minute on dry absorbent towels without dislodging the pellet. Salt washes of the pelleted DNA were then performed using 70% ethanol at a volume of 200 µl, mixing thoroughly before centrifugation at 4000 rpm for 10min at 4 °C. Again, after each spin, the 96 well plate was inverted and then spun upside down at

400rpm on absorbent towels. Two washes were performed for each sample. Plates were then allowed to air dry at room temperature for 30min prior to the addition of 10 µl of formamide (Hi Di formamide, Life Technologies, Paisley, UK). The plate was then denatured on the thermal cycler at 95°C for 5 min. Samples were incubated on ice until placing on the capillary sequencer.

	Model	Company
Sequencer	3730 DNA analyzer	Applied Biosystems
Thermo cyclers	Tetrad2 peltier thermal cycler	BIORAD
	GeneAmp PCR system 9700	Applied Biosystems

Table 7. Technical data on PCR and sequencing machines

2.3 Checking mutations

Once the capillary sequencing technique had been completed, the chromatograms were checked prior uploading onto sequence comparator programs (4Peaks online sequence analysis tool: nucleobytes.com). A visual check was made at this stage to assess the interpretability with special reference to significant dye blobs, unclear sequence and abnormally small or large traces. Where the sequence trace could not clearly be defined, the experimental technique was reviewed and repeated. Sequencing was performed in both 5' and 3' directions to act as an internal control. Where one or other sequence trace was clean enough to be appropriately checked against the reference sequence that was deemed sufficient assuming the entire length of the exon could be interpreted. Where a potential mutation had been identified, verification was sought at the corresponding position on the reverse strand. If a potential mutation was identified but could not be confirmed, the experiment was completed until a clean trace was produced.

Each case was deemed complete when all exonic regions had been assessed clearly and without cause for the possibility of a mutation.

Once confirmed on both strands, the position of the mutation was recorded and interrogated in dbSNP (National Centre for Biotechnology Information, USA) and Ensembl (Sanger Institute, Cambridge, UK) databases. Catalogue of Somatic Mutations in Cancer (COSMIC, Sanger Institute, Cambridge, UK) databases were also interrogated for mutations identified in DNA extracted from tumours. Where mutations were found to be known single nucleotide polymorphisms (SNPs), the positional numbers were recorded along with the expected frequencies.

Where confirmed mutations were found not to have been previously documented in the searched reference databases, the mutation was examined in further detail, by modelling the potential amino acid (AA) change that would likely come from the mutation and using online tools (PolyPhen 2, Harvard, USA) and SIFT (J Craig Venter Institute, USA) to model the potential damaging effect of the mutation. The modelling programmes use certain parameters to predict protein shape changes based on the availability of known data on mapped mutations as well as understanding of the hydrophobic and hydrophilic properties of the wild type and mutated amino acid sequences.

2.5 Whole exome sequencing (WES)

2.5.1 Patient selection

All exome sequencing was performed at Kings College London as part of a bespoke sequencing service and with support from Michael Simpson. DNA was extracted by the genetics laboratory at Birmingham Women's Hospital (BWH) from peripherally derived blood leukocytes from selected patients with our initial CCH/MTC pedigree family (germline DNA). A DNA shotgun library was created by DNA fragmentation and stabilisation using a standard protocol for exome sequence library generation as per manufacturer's instructions (Agilent; Santa Clara, USA). Exomes were captured using the Agilent 50Mb capture microarray. Sequencing was performed using the Illumina HiSeq 2000 platform with percentage exome capture reads to >20x depth base calling threshold (Illumina; San Diego, CA, USA). Results were expressed as a mean base depth read as well as the proportion of the exome that was captured above threshold.

2.5.2 Data filtering strategies: bioinformatics approaches

Multiple strategies are employed in WES data analysis to filter the 20,000+ expected mutations from each sample (Ng et al., 2010). The key to successful filtering strategies is the application of a logical, scientifically bio-clinical sound principle based on what is known already or can be inferred about the disease. The strengths and weaknesses of these approaches are considered in greater detail in the Chapter 5 discussion. Figures in Chapter 5 show

iterative search strategies based on a **no[1]**-novo and non-de-novo paradigm. In addition to these search methodologies, by identifying a family with rare phenotype/genotype relationship we are by default adopting a rare phenotype approach. Once candidate mutations had been identified, each was checked in turn with variant servers to identify previously described interactions and ensure novelty. Searches were performed to ascertain any known biological interactions that may be plausible with regard to MTC development. Furthermore, evolutionary preservation was recorded and mutations were ranked for direct comparison using the grading system shown in Table 8.

Characteristic	Star rating
Evolutionary preservation	*
Cancer related pathway	*
MTC related pathway	*
Mutation likely to cause significant alteration to protein structure	*
Known oncogene/tumour suppressor gene	*

Table 8. Scoring system for direct comparison of chosen mutations

2.6 Cloning and sub-cloning

Plasmid constructs containing CMV promoter regions for the transient transfection into cell lines are an established method of *in vitro* hypothesis testing. Each construct has a tagged region for identification following transfection. For each chosen construct the following open reading frames were incorporated to allow comparison of WT ER α and WT ER β a/WT ER β b ER beta isoform effects to be measured against cells transfected with vector only controls. Once wild type constructs had been created and verified by Sanger sequencing, mutations were induced in the two ER beta isoforms

using specifically designed primers and site directed mutagenesis to mimic directly the mutations in ER β c. 948delT and ER β p. V128L identified from the cohort of patients with MTC. All constructs including those undergoing mutagenesis were sequence verified by capillary sequencing prior to use in cell models as previously described.

Two specific backbones were chosen (Figure 18: Origene, Rockville, Ma, USA). Initially, pCMV6-Entry vectors were used but were problematic in experiments using the c. 948delT mutation due to suspected premature stop codon and the inability to therefore detect C terminal Myc tagged product. Wild type ER β plasmids were created using the following protocol.

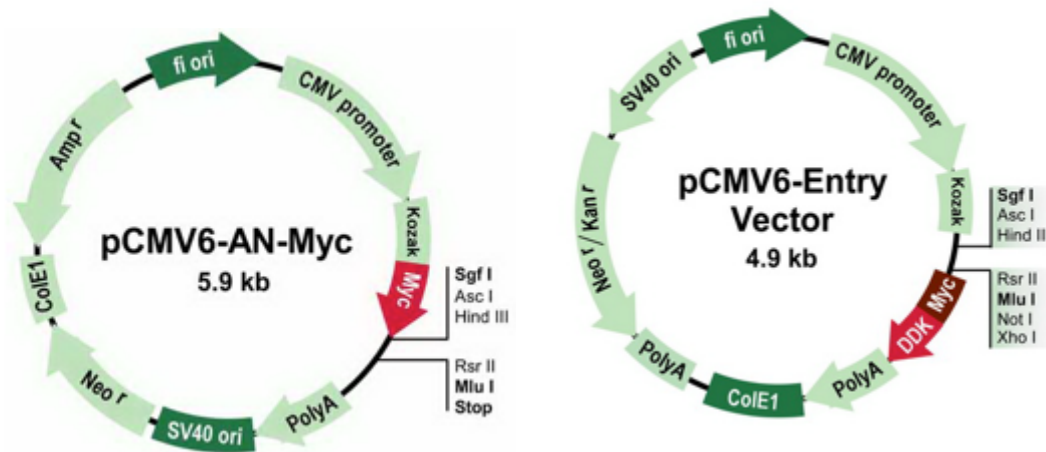


Figure 16. pCMV6 vector maps N and C terminal tagging

2.6.1 Open reading frame generation by cDNA amplification

cDNA libraries were created using reverse transcription chemistry kit (Life Technologies, Paisley, UK) from pre-held RNA libraries as per manufactures instructions. Multiple RNA libraries were searched to find a line in which ESR1 and 2 were expressed with the best being chosen for amplification. Exonic

primers were designed to amplify the appropriate section of RNA and to check the final sequence and ensure pre-existing ESR mutations were not present or induced the amplification process.

Reverse transcription 1	
Ramdon primers	0.5ul
RNA	1ul
dNTPs	1ul
dH2O	10ul
Total volume	13ul

Table 9. Reverse transcription 1

The mixture was denatured at 65°C for 5 minutes before incubating on ice for >1min, before adding reagents listed in RT2.

Reverse transcription 2	
X5 Stranded buffer	4ul
0.1M DTT	1ul
Superscript III	1ul

Table 10. Reverse transcription 2.

The reagents were mixed by pipetting

Reverse transcription cycling parameters	
Incubate 25°C	5min
Incubate 50°C	30-60min
Incubate 70°C	15min
Incubate 12°C	Forever

Table 11. Reverse transcription cycling parameters.

2.6.2 Digestion reaction

Plasmids and open reading frame were digested using complementary restriction enzymes (New England Biosciences, MA, USA) to match ESR1 and ESR2 gene created complementary products for ligation to ultimately allow ORFs to be incorporated into the plasmid. Initial digestion of both pcDNA and CMV Flag plasmids were as follows.

Digestion reaction 2 ORF for pCMV6-Entry	
Hind III	2ul
BamH1	2ul
Buffer E	4ul
DNA	30ul
dH2O	2ul
Total	40ul

Table 12. Digestion reaction 1

Digestion reaction 4 ORF for pCMV6-Entry	
Hind III	2ul
BamH1	2ul
Buffer E	4ul
CMV flag	1ul
dH2O	4ul
Total	10ul

Table 13. Digestion reaction 2

Restriction enzymes, reagents and DNA were incubated at 37°C for 4 hours. Products were separated and as such purified from unwanted DNA and reagents by gel electrophoresis and checked for correct product size against 1kb DNA ladder (Generuler, Thermoscientific, Paisley, UK).

2.6.3 Gel purification and extraction

Products were run out on 2% agarose gel blocks created as previously described using a 30ul well with equal volumes of product and DNA loading buffer. Gel electrophoresis was carried out at initially at 100mv for 10min before increasing to 180mV for enough time to adequately separate the ladder and allow an estimate of mass quantification against the 1kb DNA ladder. Once separated, the gel block was transferred to a dark room and visualised under a UV light to identify the appropriate DNA band to be

extracted. Bands were cut using a scalpel as close to the band as possible to reduce the likelihood of unwanted DNA capture and quantity of gel product to be removed by the extraction.

Gel extraction was performed using a gel extraction kit following the manufacturers protocol (Qiagen; Hilden, Germany). In brief, following band excision the fragment was weighed and added to a microcentrifuge tube with 6 volumes of supplied buffer (QG) prior to incubation at 50°C for 10min or until the fragment had completely dissolved. The mixture was vortexed periodically during incubation to ensure adequate mixing. The colour of the mixture was checked prior to proceeding. As the QG buffer contains indicator yellow at pH 7.5, orange mixture after dissolution was mixed with 10ul 3M sodium acetate pH 5.0 until the mixture resembled the colour of the initial buffer. Isopropanol was then added to the mixture to an equal v/w ratio of the initial gel block: 1ul per mg. The mixture was then placed in a micro-centrifuge into the manufacturer-supplied, DNA binding tube and spun at high speed (>10, 000 rpm) the flow-through was discarded and the step repeated as necessary until all of the dissolved mixture had passed through the membrane. Additional wash steps were then applied to the DNA binding column to remove traces of agarose and salts that may impair further reactions, each time spinning at high speed. Finally, the DNA was eluted in to a clean micro-centrifuge tube using 30ul of 10mM TRIS-CL, pH 8.5 placed directly on the membrane for 1 min prior to high speed centrifugation for an additional minute. DNA concentrations of the eluted product were measured using the

Nanodrop® ND-1000 spectrophotometer on DNA settings (Thermo Scientific, Willmington, DE, USA).

2.6.4 Ligation reaction

Purified plasmids and open reading frames (ORF) were ligated under the following conditions. Ligation conditions were calculated (quantity of ORF in ng was calculated for a 1:1 ORF to plasmid ratio) using the following equation.

$$\frac{\text{Insert length (base pairs)} \times \text{Vector mass (ng)}}{\text{Vector Length (base pairs)}} = \text{Insert mass (ng) 1:1 ratio}$$

Although it is generally recommended that an ORF to plasmid ratio of 3:1 is optimal for ligation, conditions were optimised by running multiple experiments and controls with varying ratios of ORF to plasmid. Each combination of ORF to vector was run in the ratios 1:1, 1:2, 1:3 and 1:6.

Ligation reaction	
T4 ligase	1ul
Buffer	1ul
dH2O	Up to 10ul
DNA insert	Variable depending on ratio
DNA vector	Variable depending on ratio

Table 14. ligation reaction

2.6.5 Bacterial transformation

Silver competent cells were used to transform ligated ORF containing plasmids for maxi preparation under the following conditions. 10ul of ligated product was mixed with 100ul of DH5 α (silver competent e coli cells thawed for -80 on ice for 20min: Life Technologies, Paisley, UK). Plasmids and bacteria were gently mixed by swirling and incubated on ice for 30min. A heat shock was performed to encourage bacterial uptake of plasmid using a heat block set at 42°C for 45 sec. The mixture was then incubated on ice for 2 min prior to adding 500ul of prewarmed super-[optimised](#)^[2] media (SOC, Sigma Aldrich: St Louis, USA) 20g/l Tryptone, 5g/l yeast extract, 4.8g/l MgSO₄, 3.603g/l dextrose, 0.5g/l NaCl, 0.186g/l KCl) for transformation and incubating at 37°C for 60-90min. The mixture was pelleted by gentle centrifugation at 1000rpm for 1min. The supernatant was removed and the pellet re-suspended in 150ul of SOC media prior to plating and spreading on pre-prepared, prewarmed Luria Bertani (LB) agar (Sigma Aldrich: St Louis, USA) plated with Ampicillin at a ratio of 1:1000. Plates were incubated overnight at 37°C.

After overnight incubation, colonies were picked using a sterile pipette tip and placed in 2mls LB broth with ampicillin 1:1000 and incubated overnight in a rotatory incubator at 220rpm at 37°C to create a starter culture.

Following overnight incubation, 1.5ml of starter culture was harvested for mini prep and sequencing to verify the ORF, promoter region and ensure no inadvertent mutations had been induced. Once confirmed the cultures

containing the correct sequence ORF and promoter regions were selected for maxi prep and purification.

2.7 Site directed mutagenesis

Once wild type plasmid backbones had been established and checked by capillary sequencing, site directed mutagenesis was performed using a standard kit (Agilent II XL SDM, Santa Clara, USA) to induce the mutations c. 948delT and p. V128L. The kit uses a process of PCR amplification with primers specifically designed to induce to desired mutation. Following plasmid dsDNA denaturing, mutagenic primers are annealed to the template backbone and extended using DNA polymerase. Dpn I endonuclease is then used to digest parental template DNA by digesting methylated and hemi-methylated DNA thus selecting for non-mutated DNA.

Specific primers were designed using the following protocol as recommended by the manufacturer for SDM primer design. The primer designing software, Primer X, web-based package was used to create a primer pair with 10-15 bases of normal sequence either side of the mutation (deletion or single base change)(Primer X; bioinformatics.org). Primers were designed to anneal to either strand of the ORF with its plasmid backbone such that the mutations were in the same position. Recommendations for primer design include primers of 15-25bp length to minimise the risk of secondary structure creation and melting temperature of $>78^{\circ}\text{C}$. Primers designed on Primer X were checked against the following equations for single base deletion and base

substitution respectively where the value N is the length of the primer in base pairs as previously described. Further, primers with 10-15 bp either side of the mutation and >40%GC content ending in multiple C or G bases are favoured. Primers were purified prior to use using polyacrylamide electrophoresis (PAGE) to increase annealing efficiency.

$$T_m = 81.5 + 0.41(\%GC) - (675/N)$$

$$T_m = 81.5 + 0.41(\%GC) - (675/N) - \%mismatch$$

A control reaction was set up as per the manufacturers instruction using the supplied control plasmid and predesigned primers. The control allows an assessment of the success of cycling and digestion stages of the experiment colony assessment and picking.

Site directed mutagenesis reaction	
10 X reaction buffer	5ul
dsDNA template	15-45ng (1-3ul)
Forward primer	125ng
QuikSolution	3ul
Reverse primer	125ng
dNTP mix	1ul
dH2O	Up to 25ul

Table 15. Site directed mutagenesis

1ul (2.5U/ul) of Pfu Ultra HF DNA polymerase was then added

In the control sample, the control plasmid primers were substituted and cycling parameters adjusted to plasmid specific length to enable adequate extension.

SDM cycling parameters	Cycles	Time
Incubate 95°C	1	1 min
Incubate 95°C	1	50 sec
Incubate 60°C	1	50 sec
Incubate 68°C	1	1 min/kb plasmid length
Incubate 68°C	1	7 min
Incubate on ice for 2 minutes following thermal cycling		

Table 16. Site directed mutagenesis cycling parameters

2.7.1 Amplification of selected plasmids to verify sequence and for use in in-vitro work (mini and maxi prep.)

In order to determine which of the selected colonies contain the correct plasmid and insert and to ensure that additional mutations had not been introduced during the cloning process, DNA purification was performed from overnight colony cultures. Purified DNA was then sequenced using previously described methods. DNA purification was performed using a kit designed for use with a micro-centrifuge using the manufacturers protocol (Qiagen Spin Miniprep)(Anon, n.d.). In brief, starter cultures were created by picking a single colony from an antibiotic selective agar plate incubated for 18 hours as previously described. After overnight incubation, colonies were picked using a sterile pipette tip and placed in 2mls LB with ampicillin 1:1000 and incubated overnight in a rotatory incubator at 220rpm at 37°C to create a starter culture. Bacterial cells were harvested (from 1ml of starter culture) by centrifugation in a 1.5ml bench top microcentrifuge tube at 8000rpm for 3min. The remaining starter culture was catalogued and stored at 4°C until sequencing had been completed and to provide starter culture for maxi preparation if confirmed successful.

Pelleted bacterial cells were re-suspended in 250ul of re-suspension buffer (P1) including RNase and mixed to ensure no clumping of bacterial material. The bacterial cells were then lysed with an equal volume of lysis buffer (NaOH/SDS: buffer P2) to release cellular contents including chromosomal and plasmid DNA, RNA and cellular proteins all of which are denatured. Following neutralisation with 350ul of buffer N3, which contains high salt and enables plasmid DNA to renature whilst other cellular debris including chromosomal DNA to precipitate, the solution was placed in micro-centrifuge at high speed (13,000rpm) for 10min. The precipitated lysate material forms a white pellet with plasmid DNA remaining in suspension in the supernatant. The supernatants were then added to the silica containing spin tube and plasmid DNA adsorbed onto the membrane by centrifugation at high speed for 1 min. A series of wash steps were then performed to remove nucleases and reduce the salt content prior to elution as per manufacturer's instructions. Finally, the purified plasmid DNA was eluted into a clean micro-centrifuge tube using 50ul of 10mM Tris-Cl, pH8.5, & left standing for 1 min prior to centrifugation at high speed for 1 minute.

2.7.2 Maxi preparation and sequencing

Once the starter culture integrity had been confirmed by sequencing of plasmid^[3] DNA purified using the mini prep technique outlined (Sigma Aldrich: St Louis, USA). Maxi preparation of plasmids works on a similar principle to mini preparation including, alkaline lysis, precipitation of non-plasmid lysate products and plasmid DNA binding by resins which can then be washed and desalted prior to elution. In brief, starter culture as previously described was

diluted to 1 in 500 in 100ml antibiotic selective LB media in a large conical flask Ampicillin 1 in 1000). The flask was incubated overnight at 37°C for 16 hours, with vigorous shaking at 220 rpm. Cells were harvested from the media by centrifugation at 6000 x Gravity (G) for 15min at 4°C and decanting and discarding the supernatant. Cells were re-suspended in 10mls of the manufacturers buffer (P1) containing RNase in a 50ml flacon tube. Lysis of the bacterial cells to release the cellular contents was achieved by adding 10ml of lysis buffer (P2) and gentle mixing to avoid inadvertent chromosomal DNA shearing that could contaminate the purified plasmid. Precipitation of chromosomal DNA, RNA and cellular proteins was achieved by gentle mixing of 10mls of chilled precipitation buffer after which a floating white material could be observed. The supernatant was separated from the precipitated product by centrifugation at 20,000 x g for 4°C.

The plasmid binding resin column was prepared by applying 10mls of the equilibration buffer and flow to be driven by gravity. The supernatant was then added to the activated resin and allowed to flow through by gravity. Two salt removing wash steps were then applied to the bound plasmid DNA prior to elution in 15mls of manufacturer supplied elution buffer (buffer QF). The DNA was then further precipitated by adding 10.5mls of isopropanol prior to centrifugation at 15,000 x g at 4°C for 30min. The supernatant was removed and discarded prior to the pellet being washed in 5ml of 70% ethanol centrifuged for 10min at 15, 000 x g. after decanting and discarding the supernatant the DNA pellet was air dried for 10min and eluted in 10mM Tris-

Cl, pH 8.5. Concentration was determined using spectrophotometry as previously described.

2.8 Cell culture

MCF7 cells were kindly supplied by Professor CJ McCabe (School of Clinical and Experimental Medicine, UoB, UK). MCF7 cells maintained in RPMI 1640 media (Sigma-Aldridge, Poole, UK) supplemented with 1% Pen/Strep (Penicillin/Streptomycin: Invitrogen, Paisley, UK), 1% L-Glutamine (Life Technologies, Grand Island, NY, USA) and 5% Fetal Bovine Serum (FBS) and passaged twice weekly. T47D were maintained in Delbecco's modified Eagles Media (DMEM) supplemented with 10% sterile FBS 1% Pen/Strep and 1% L-Glutamine. All transfection experiments were carried out between passage 20 and 40 after which cells were discarded. Cells were maintained as a monolayer in T75 flasks at 37 °C in an incubator with 5% CO₂. On passage, the media was removed and cells washed with 5mls of sterile PBS. Cells were trypsinised with 1ml 0.25% trypsin and incubated for 5 minutes at 37 °C prior to being loosened and re-suspended in media. Cell counts were performed using cell counting slides. Cells were seeded at a concentration of 150,000 and 75,000 cells per well in 6 and 12 well plates respectively. Optimum seeding count varied between experiments although cells were only transfected on reaching 50-60% confluence under the following conditions. TT cells were maintained in Ham's F12 media (Life Technologies, Grand Island, NY, USA) supplemented with 10% sterile FBS but were otherwise managed as pre MCF 7 cells.

Reagent/plasmid	
LT1	6ul
Optimem	200 μ l
Plasmid	2 μ g

The transfection reagent LT1 (Mirus Bio, Madison, WI, USA) was allowed to reach room temperature for 20 minutes prior to use as per manufacturers guidelines (Anon, n.d.). Reagents were mixed gently in 1.5ml centrifugation tubes for 25 minutes in sterile conditions prior to transfection. Transfection reagent containing media was changed for fresh media 5 hours post transfection. To lyse and harvest, the media was removed and washed with sterile PBS. Cell lysis and protein harvest was performed using 150 μ l radioimmunoprecipitation assay (RIPA (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0)) buffer supplemented with a premixed cocktail of protease inhibitors to specifically inhibit serine, cysteine and aspartic proteases, and amidopeptidases (6%: 1.5 mM Pepstatin A, 104 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) 80 μ M Aprotinin, 1.4 mM E-64, 2 mM Leupeptin, 4 mM Bestatin, in dimethyl sulfoxide (DMSO).

Cell plates were incubated at -20°C for a minimum of 30min. Lysed cells and protein were harvested from the cell plate using a rubber policeman and the solution transferred to a clean micro-centrifuge tube prior to centrifugation at 13,000 RCF at 4°C for 10 minutes. The supernatant was decanted into a clean micro-centrifuge tube and the pellet consisting of cellular debris, discarded.

2.9 Western blotting

2.9.1 Preparation of polyacrylamide gel for protein electrophoresis

Corresponding glass slides and gel combs were selected to produce a polyacrylamide gel 2mm thick (SDS-PAGE). Western blotting apparatus (BioRad) were set with glass side holders up and tested for leaks by filling with water. 12% polyacrylamide gels were used in all experiments. The resolving gel was prepared using the recipe in table 1 with Tetramethylethylenediamine (TEMED) and APS (ammonium persulphate) which, together, catalyse the polymerisation of acrylamide. TEMED and APS are added just prior to pouring to allow sufficient time to pour all gels before the acrylamide polymers form and the gel sets. The quantities shown in table one were sufficient for two gels. Resolving gels were poured between glass slides to a point just below where the comb sits when fitted. Once poured, 50% methanol was added to the top of the gel prior to setting to encourage an even spread of gel and remove any bubbles which may affect the direction in which the proteins migrate during electrophoresis. Resolving gels were left in the apparatus at room temperature for 30 min. Stacking gels were made using the recipe in table 18 and 19, again omitting the TEMED and 10% APS until the end to prevent premature setting of the gel. The methanol was removed from the top of the resolving gel and the stacking gel poured on top prior until the recess between the glass slide was full. The comb was then placed into the non-set stacking gel and any excess displaced gel removed. Again, the gel was left at room temperature for 30 min to fully set. Once set the combs were removed and the wells rinsed with 1 X running buffer and checked for integrity prior to protein loading.

Acrylamide resolving gel for western blotting (12% gel)	
dH ₂ O	4.935ml
1.5M Tris-HCl pH 8.8	3.75mls
Acrylamide	6ml
10% SDS	150ul
TEMED	15ul
10% APS	150ul

Table 17. Western blotting 1

Acrylamide stacking gel for western blotting (5% gel)	
dH ₂ O	2.71ml
0.8M Tris-HCl pH 6.8	1.25mls
Acrylamide	833ul
10% SDS	100ul
TEMED	10ul
10% APS	100ul

Table 18. Western blotting 2

2.9.2 Protein preparation and loading

Protein concentration was estimated using the bicinchoninic acid (BCA) colorimetric assay kit (Pierce) where protein from each experiment is compared against a reference standard using a spectrophotometer or microplate reader (Victor 3 1420 Multi-label counter, Perkin Elmer). Coomassie Brilliant Blue G-250 dyes bind to protein in a stable manner altering the wavelength of light absorbance from red in acidic conditions ($\lambda_{\text{max}} = 470 \text{ nm}$) to blue when protein bound ($\lambda_{\text{max}} = 595 \text{ nm}$) in which form it can be detected. In ideal systems, the reference standards are purified and calibrated versions of the protein of interest however in the absence of these, we used purified bovine serum albumin (BSA) as a surrogate standard against which to measure our extracted protein.

Protein estimation was performed using duplicate wells of protein extracted from each experiment compared against 10 duplicate reference standards. The standards ranged from 0.0 to 5.0mg/ml of BSA and were arranged in a 96

well plate suitable for analysis in a micro plate reader (Wallec: 560 nM absorbance setting). In each well, 80 ul of manufacturers solution (Buffers A and B in a ratio 50:1) was added followed by a further 4ul of either protein standard BSA or protein extracted from cell lysis. Once the protein had been added the plate was incubated at 37°C for 30 min prior to placing on the plate reader. By plotting the standards absorbance against concentration, a standard curve can be generated against which experiment protein concentrations measured. By plotting the absorbance of the sample protein, an estimate of protein concentration can be made. Using [these](#)^[4] estimates of concentration allows an equal quantity of protein to be loaded in each well prior to electrophoresis such that ultimately, quantitative measures of relative protein expression can be made between differing experiment conditions.

The set gels were transferred to electrophoresis chamber and the dam filled to the level specified by the number of gels with 1 X running buffer. 15-30 ug of protein was denatured by incubating with loading buffer (lamelli buffer: Biorad with DTT (50mg/ml) at 95°C for 5 min. Samples were then mixed with loading buffer and loaded into the appropriate wells in the polyacrylamide gels. A protein ladder was used to confirm presence or absence of proteins of the appropriate size (Precision Plus Protein Standards: Biorad). The apparatus was topped up with running buffer and the apparatus run at 70mV until the samples and ladder had progressed through the stacking gel. Once in the resolving gel the voltage was increased to 110mV-130mV and run until the loading dye reached the bottom of the gel.

2.9.3 Protein transfer onto nitrocellulose membrane

Polyvinylidene difluoride (PVDF) membranes were cut to the appropriate size and activated with 100% methanol for 30s prior to use. Transfer cassettes were loaded with fibre pads, filter paper, polyacrylamide gel, PVDF membrane, filter paper and further fibre pads. All contents were thoroughly soaked in transfer buffer prior to use. The cassettes were closed and placed in the transfer apparatus. The apparatus was filled with transfer buffer and cooled by adding an ice block before running for one hour at 350mA. In this time protein from within the gel is able to pass down an electrochemical gradient and onto the PVDF membrane which avidly and non-specifically binds protein and will display on probing, proteins in the relative position as they were in the gel. Once transfer had completed, the membranes were blocked with TBS-T 5% non-fat milk (Marvel) for 2 hours. TBS-T was replaced with TBS-T 5% milk and primary antibody at a concentration specified in each experiment. Blocked membranes were incubated with primary antibody on a mobile agitator overnight at 4°C.

After overnight incubation with the primary antibody (specific to the protein of interest), the membrane was washed with TBS-T for 15 min on a mobile agitator for 1 hour with four changes of TBS-T during that time. The membrane was then incubated at room temperature with secondary antibody (specific to the primary antibody and containing detectable horseradish peroxidase: HRP) in 5% milk TBS-T for 1 hour. Further washing for one hour was then performed, washing with TBS-T with changes every 10 minutes (six in total).

Running buffer	
dH₂O	4.935ml
1.5M Tris-HCl pH 8.8	3.75mls
Acrylamide	6ml
10% SDS	150ul
TEMED	15ul
10% APS	150ul

Transfer buffer	
dH₂O	4.935ml
1.5M Tris-HCl pH 8.8	3.75mls
Acrylamide	6ml
10% SDS	150ul
TEMED	15ul
10% APS	150ul

2.9.4 Developing nitrocellulose membranes following antibody incubation

Membranes were developed using ECL plus (Pierce: Thermo Scientific) as per manufacturers recommendations (Anon, n.d.). In brief, membranes were face up on sheets of cling-film and patted dry with paper towels. ECL reagents, which, when combined react with HRP to produce a chemiluminescence detectable on exposure to camera film, were premixed in a v/v ratio Buffer A: buffer B = 40:1. Approximately 500ul of the mixed ECL solution was applied to each membrane and gently spread with a pipette tip to ensure even spread and remove bubbles. The membranes were incubated at room temperature for 5 min after which excess developing solution was removed and the membrane secured and sealed in a clear plastic wrap. Membranes were developed in a dark room using Xray film (Kodak Biomax, Sigma Aldrich: St Louis, USA) and chemical developer. The length of

exposure between the membrane and the film was varied using a standard protocol tailored to the individual developing characteristics of each membrane.

Once developing of the protein of interest was complete, the membranes were washed as previously described and then re-probed for Beta actin (primary antibody) and the appropriate HRP conjugated secondary antibody (see table). As Beta actin binding was so strong, re-probing membranes without first stripping and re blocking caused no problems with interpretation.

Primary antibodies	Manufacturer	Animal
ERα	Abcam (ab37438)	Rabbit
ERβ	Abcam (ab288)	Rabbit
MYC	Cell signalling (2272)	Rabbit
RET	New England Biolabs (C31B4)	Rabbit

Table 19. Antibodies for western blotting

2.10 Immunofluorescence

Cells were cultured and transfected under standard conditions as previously described. The media was removed and cells were washed with 1ml sterile PBS. Cells from 6 well plates were fixed in 800ul of fixing solution (see table 21) and incubated at room temperature for 20min.

Fixing solution for Immunofluorescence	
0.2M NaHPO₄ corrected to pH 7.4 (Corrected with 0.2M NaH₂PO₄)	20ml
2% PFA	0.8g
2% Glucose	0.8g
0.1% sodium azide	8mls
dH₂O	12mls

Table 20. Fixing solution for immunofluorescence

Once fixed, cells were rinsed twice with sterile PBS. To make cells more permeable, 800ul methanol (100%) was added to each well and left to incubate at room temperature for 10min. Two further washes with sterile PBS were then performed prior to blocking with 800ul of 10% NCS (in PBS) for 30 min. 80ul of primary antibody was prepared with 80ul 1% BSA (in PBS) for each coverslip. An aliquot of the solution was then added to parafilm and each coverslip lowered face down onto the antibody. Antibody treated coverslips were incubated at RT for 1 hour in the dark. Cover slips were returned to 6-well plates and rinsed three times with sterile PBS.

Secondary antibodies (1:1250) were prepared in 80ul aliquots of 1% BSA (in PBS) with 1% NCS (Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG: Invitrogen). Hoescht stain was then added at 1:1000. 80ul aliquots of solution were added to parafilm and coverslips lowered onto the solution. Incubation for 1hr at RT in the dark was then performed. Coverslips were then returned to 6 well plates prior to washing with sterile PBS three times. Coverslips were blotted prior to mounting on slides using Dako Fluorescent Mounting Medium and left to dry in the dark for 2hours at 4°C.

2.11 mRNA Expression

Cells were maintained and passaged as previously described. To measure mRNA expression real time chemistry was employed. RNA was extracted following 24-48 hour transient transfection using an RNA extraction kit (RNeasy: Qiagen, Hilden, Germany). In brief, cells were cultured in 12 well plates to avoid having too much RNA for the extraction technique ($<10^7$ cells). Cells were lysed in situ using the manufacturers buffer (RLT) and ethanol in volumes approximated to the number of cells present in each well.

To release the maximum quantity of RNA from cells and organelles, lysates were disrupted and by passing through a homogeniser in a microcentrifuge at high speed for 2 min (QIAshredder, Qiagen, Hilden, Germany). Once homogenised, 1 volume of 70% ethanol was added and mixed by pipetting prior to transfer to a RNA purifying microcentrifuge column, centrifuging for 15 sec at $>8000 \times G$. The flow through was discarded and the silica-trapped RNA in the spin columns was washed as per protocol using the supplied buffers at the described spin speeds to initially ethanol purify and then ethanol remove from the sample. Once complete the RNA was eluted into a clean micro centrifuge tube in nuclease free water and stored at -80°C until required.

RNA concentration from each experimental condition was measured using the Nanodrop ND-1000 spectrophotometer on RNA settings prior to reverse transcription. Sterile H_2O was added to 0.5ug RNA to a total volume of 4.9ul. Samples were incubated at 70°C for 10 minutes prior to pulse spinning and incubation of ice. A mastermix for RT-PCR was prepared using manufacturers

supplied products and mixed gently by pipetting adding the RNasin and AMV last and keeping on ice throughout (Promega; Madison, WI USA).

RT PCR master mix	
MgCl₂	2ul
10xRT buffer	1ul
dNTPs	1ul
Random Primers	0.5ul
RNasin	0.25ul
AMV	0.3125

An aliquot of 5ul of master mix was added to each RNA sample and incubated at room temperature for 10min prior to placing on PCR block under the following conditions.

RT PCR	
1 hour	42 ⁰ C
5 min	95 ⁰ C
5 min	4 ⁰ C

The samples were pulsed and spun again prior to adding 30ul of H₂O to give a final concentration of cDNA approximately 12.5ug/ul. Samples were stored at -20°C prior to use. 25ng of cDNA was used in each real time assay (see Chapter 7).

2.12 Immunohistochemistry

Immunohistochemistry was carried out on our behalf by the pathology labs at UHB using an automated system as per manufacturer's instructions (Leica Bondmaz Immunostainer; Protocol F). 10 micron slides of tumour and normal tissue were cut for immunostaining and also for FFPE DNA and RNA extraction. Antibodies for immunohistochemistry were optimised prior to using on study tissue using breast and uterine controls. Antibodies (Novocastra Lyophilized Mouse Monoclonal Antibodies) were purchased from Leica

Microsystems. Tumour and normal slides were identified by re-examination of the H and E stained slides from corresponding blocks. Samples were deparaffinised by DEWAX using manufacturers protocol prior to rehydration with PBS. Samples were then immersed in distilled water.

In brief, the manufacturers protocol includes a 20 minute heat induced epitope retrieval stage, undertaken at 90°C with an EDTA based buffer (Ph 9). Peroxidase was then added and incubated for 10 minutes to remove endogenous peroxides that may induce false positives. Once completed the slides were washed again in PBS.

A set volume of 150µl of primary antibody ERbeta was added as per manufacturer's instructions with an optimised dilution ratio of 1:50 using manufacturer's antibody diluent. The antibody was then incubated on the slide for 15 minutes prior to two washings with PBS. Each wash step was performed for 5 minutes. The slides were then incubated with a mouse secondary antibody conjugated with horseradish peroxidase for 10 minutes. Again, slides were washed twice with PBS for 5 minutes per wash.

2.12.1 Chromogenic reaction

Slides were incubated in DAB after which the slides were washed in distilled water and then counterstained with Hematoxylin for 5mins. Once counterstained the slides were dehydrated in 75%, 80%, 95% and then 100% ethanol. Slides were then cleaned in Xylene prior to a cover slip being applied using DPX.

Chapter 3.

Project design recruitment and collaboration

3.1 Introduction

Although specific data on the incidence of MTC is not freely available on any of the public access cancer registries, the estimated incidence of MTC is around 3-5 per million or approximately 120 new cases per year in the UK. In the US there have been significant inroads into improving the investigation and research opportunities for people with rare or orphan diseases, defined as diseases with incidences <1 in 200 000 population and as such not commercially viable for drug development (Brewer, 2009). Significant funds have been made available and in addition, collaborations aiming to bring together researchers and patients (Wellman-Labadie and Zhou, 2010).

Rare diseases pose specific problems in terms of research, not least in attracting funding for research and development of new therapeutics. Indeed, government funding is often required in such areas due to a lack of commercial interest (Commissioner, n.d.). The involvement of governmental organisations and well as governmental policy has, led to significant improvements and opportunities for research in rare disease, especially in collaboration with patient advocacy groups (Groft and Rubinstein, 2013). In the study of rare diseases, including this study, recruitment and strategies of data collection and capture are of vital importance if clinical data are to reach an appropriate numerical level to allow intra-cohort analysis.

In the study of rare disease a number of well described methods are available for maximising the impact of research projects both in terms of their overall ability to draw conclusions and in applicability of those conclusions to the

populations being studied (DeWard et al., 2014). Firstly, rare diseases often have specific medical and surgical needs, access to which is not available in every centre. Using MTC as an example, patients often present with complex surgical needs or need revision surgery for recurrent disease; evidence supports improved outcome in these patients if managed in larger centres with high throughput (Smith, Watkinson and Shaha, 2012). Furthermore, the complexity of MTC with regard to the genetics and contact tracing^[5] again can usually only be supported in larger centres with access to regional genetics services (Groft, 2013). As such, many of these rare conditions coalesce naturally towards larger or tertiary centres and within those centres towards individual practitioners and small collaborating groups (Richard Gliklich and Leavy, 2011). The head and neck surgical department and clinical genetics department collaboration for MTC and skull base tumours in Birmingham are an example of this.

By identifying individual clinicians with a special interest in the disease it is possible to capture a large number of patients who are often regular attendees to follow-up and review clinics in these centres. MTC by its nature requires long-term or even lifelong follow-up with calcitonin monitoring and in some cases, serial imaging to monitor treatment response and recurrent disease (Kloos et al., 2009). The added advantage of approaching clinicians in tertiary centres is that patients under long-term review by clinicians with a special interest in their disease often have good relationships with their practitioners and as such are well motivated to take part in research project that may directly or indirectly benefit them (DeWard et al., 2014). Secondly,

practitioners with special interest often maintain databases of patients treated by their department and as such may have further access to patients not routinely seen in clinics or whom their local departments follow routinely (Richard Gliklich and Leavy, 2011). Access to databases as a point of contact has the potential to allow large numbers of patients to be contacted at a single point in time rather than recruiting through direct contact in the clinical setting.

As well as medical practitioners and patient databases held within hospital departments, patients with medical issues and in particular, rare diseases are increasing forming their own groups and linking with charitable organisations. Patient advocacy groups have helped shift the debate and focus governmental departments and researchers towards their needs (Dunkle, Pines and Saltonstall, 2010). Often these groups form research registers and put themselves forward to be involved and studies have reported success in recruitment by accessing patient lead databases (Richard Gliklich and Leavy, 2011). As a self-selected highly motivated group they can be useful point of contact for recruitment as well as dissemination of information regarding research around their own communities. The facilitation of interest in these groups has been made easy with the advent and uptake of social media and indeed some studies have recruited using only these methods (Schumacher et al., 2014).

Finally, collaborations with groups across the globe have become easier and many groups have stored samples collected from previous projects that are amenable for analysis in subsequent projects. Forming collaborative links at a

global level allows projects to gain numbers quickly as well as widening the potential applicability of the results (Gupta, Bayoumi and Faughnan, 2011). In genetic terms it may help strengthen mutation searches by looking at DNA obtained from more variable and distantly related populations. Furthermore, collaboration on single projects often opens avenues to investigate other disorders and establishes a foundation for ongoing communication.

3.2 Project design

This project aims to use exome sequencing (WES) technology to identify novel disease predisposing genes within the context of MTC. Although our general hypothesis is that genes other than RET predispose to MTC, additional hypotheses regarding the nature of any discovered causative links will be subsequently generated by our exome sequencing data. To be able to test any generated hypothesis, or to add weight to a theory, we need to be able to draw on a cohort of individuals with both familial and sporadic disease as well as be able to examine germline as well as somatically derived DNA. Individuals who are most likely to have inherited a disease predisposing mutation are those with familial disease or sporadic cases where disease is diagnosed at a young age (<40 years old at diagnosis). The project design is to identify using WES in an index familial non-RET MTC to a panel of candidate germline mutations. Identified genes can then be interrogated in more detail in other families with non-RET MTC as well as individuals with sporadic disease. If other mutations are found within the same genes within these two comparator cohorts, this would provide sufficient evidence to take that mutation forwards for further interrogation *in vitro*. This method of

hypothesis generation has been shown to be successful in identifying novel candidate genes in a number of diseases including kidney cancer and pheochromocytoma (Comino-Méndez et al., 2011).

The first aim of the project therefore was to locate patients with familial non-RET positive MTC and sporadic young onset disease. We know these are likely to be difficult to find and as >95% of families with MEN2 have a RET mutation and >85% of FMTC (a rarer clinical entity) also have disease predisposing RET mutations (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014c). In addition to this, our understanding of the role of disease predisposing mutations in cancer is best exemplified by breast cancer where the BRCA1/2 genes make up the majority of disease causing mutation in families with breast cancer predisposition and the remaining disease causing mutations consist of multiple mutations of different genes in relevant pathways, each making up just a small proportion of the heritable disease (Sapkota, 2014)(Lee and Ang, 2014). Clearly, when dealing with a disease such as MTC that is several orders of magnitude rarer than breast cancer, the likelihood of finding multiple families that can be recruited to the study and the chance that those families harbour the same genetic alteration based on the experience with breast cancer is significantly reduced. If however, the same mutation were found in unrelated families, within this context, it would serve as good evidence that that mutation were indeed disease predisposing and the same would be true of patients with young onset sporadic disease.

3.3 Results

3.3.1 RET negative index familial disease

This study was conceived and followed through by the identification of the following family with MTC, CCH pedigree (Figure 18). Disease in the family was identified incidentally when a neck mass was identified in the proband aged 33, who on attendance to the Accident and Emergency department following a road traffic accident. Investigation of the neck lump revealed a thyroid tumour which was later found to be medullary thyroid cancer on diagnostic lobectomy. The patient went on to have completion thyroidectomy, lateral and central compartment nodal clearance. RET testing (sequencing) performed at the time of exons 10, 11, 13-16 was found to be negative and as such disease was presumed to be sporadic. As the proband has a monozygotic twin, investigation was offered in the form of clinical testing, calcitonin and pentagastrin stimulation testing with no nodular thyroid disease and no overt nodal disease either clinically or on radiological parameters. The proband's twin was found to have elevated baseline and stimulation calcitonin and was offered treatment or surveillance. He opted for surgery and was found to have C cell hyperplasia with no evidence of frank MTC or nodal metastasis.

Based on the finding in the proband twins, cascade testing in the form of baseline calcitonin and pentagastrin stimulation was offered to all of the offspring of the twin on the basis of increased likelihood of an inherited predisposition to CCH/MTC rather than another environmental factor. Ultimately, based on clinical testing, elevated calcitonin at baseline or on

pentagastrin stimulation and parental preference, all of the five offspring of the twins had a prophylactic thyroidectomy before the age of ten.

All histological slides were reviewed by me and a blinded, independent pathologist with a special interest in head and neck oncology (Rachel Brown, RB). Blocks were re-cut and re-stained where necessary. There was divergence from the initial classification in two of the proband's offspring with one case of CCH identified in one prophylactic specimen previously classified normal and normal histology classified in a specimen previously defined as CCH. The explanation for this lies in the subjective nature of CCH confirmation as further discussed in Chapter 7. more important in this case was the nature of the initial histological examination that is, the ruling out of MTC rather than closer inspection and diagnosis of CCH. Finally, Calcitonin staining was not routinely used in the initial staining and assessment of these specimens, partly again as the premise of the examination was to rule out MTC rather than ruling in CCH, which clearly makes a significant difference in the identification of the disease.

It should be noted also that on cascade testing the parents of the proband were also offered calcitonin and pentagastrin stimulation and the father had an equivocal stimulation test with mildly elevated calcitonin but normal imaging and chose not to pursue any further treatment.

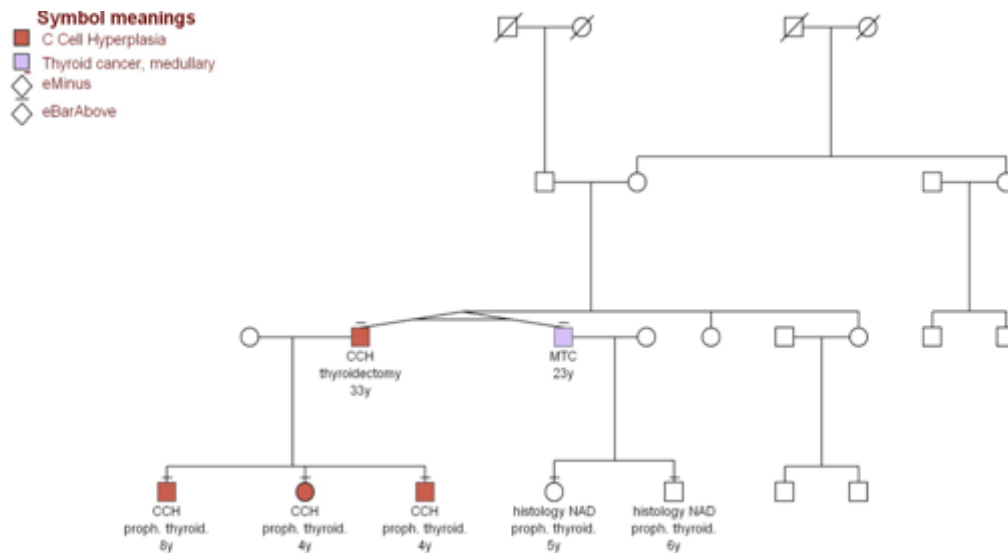


Figure 17. The index family. Proband presented age 22y with a medullary thyroid carcinoma. Following clinical work-up (abnormal calcitonin following pentagastrin (PG) stimulation (basal calcitonin 14.0, stimulated 105-Neil Gittoes QEH)). Initially the PG stimulation had been normal in 1992 and 1998), his monozygotic twin brother, underwent a total thyroidectomy age 33y with level 6 neck dissection and C Cell hyperplasia was noted on histological review. In view of the diagnoses in these two individuals, the 5 offspring were tested and opted to go on to have total thyroidectomy. From left to right, Child 1 had total thyroidectomy in 2008 age 8y – CCH present. Child 2 had some abnormal PG stimulation results so total thyroidectomy in 2006 age 4y – CCH. Child 3 had total thyroidectomy in 2008 age 4y – CCH. Child 4 had equivocal (slightly elevated basal, equivocal following PG stimulation) results in 2006 age 5y, so decision made to have total thyroidectomy in 2007 – No CCH/MTC. Child 5 Total thyroidectomy in 2010 age 6y – No CCH/MTC

3.3.2 Recruitment strategy

Based on the adopted strategy for recruitment of a comparator cohort, patients were recruited directly and other tissue samples accessed by direct collaboration. In terms of direct patient recruitment, 54 patients have been recruited to date with the majority (48%) coming through direct referral from physicians within the UK with local data base searches (28%) and patient advocate groups (24%) making up the remainder (Figure 19). 110 DNA samples were available in the wider cohort, the majority of which were acquired through collaboration with groups in Australia and Spain (42%). A significant proportion of tissue samples came through direct referral

mechanisms with direct UK clinical referral being the most frequent (28%) (Figure 20). The breakdown of these samples into germline DNA and DNA extracted from fresh or formalin fixed paraffin embedded tissue as well as the relative frequencies in each sub-group of familial and sporadic disease is shown in Figure 21. Two additional families with MTC/CCH pedigree were identified through collaborations with the Madrid group and included in this study (Figure 22). In addition, further families with MTC/CCH pedigrees have been identified through the advertisement and recruitment strategy, although they were identified too late in the process to have been included for further analysis here (Figure 23).

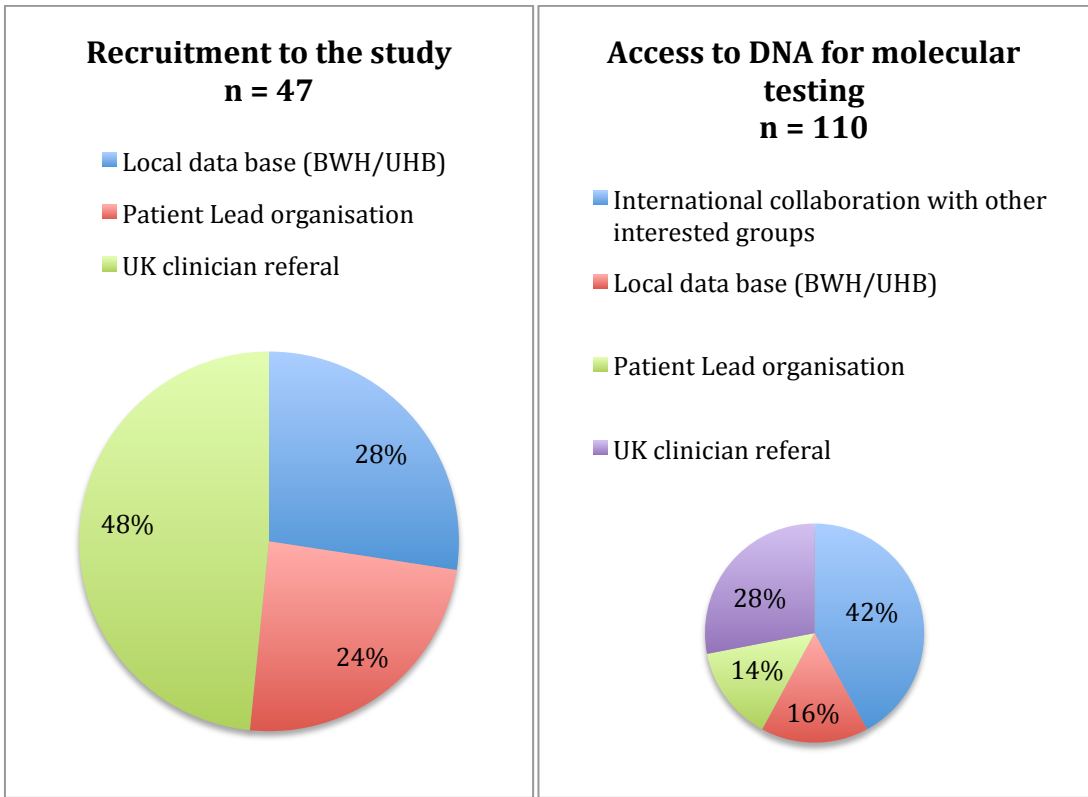


Figure 18 and Figure 19. The relative frequencies of patients and DNA/tissue available from entry point into the study

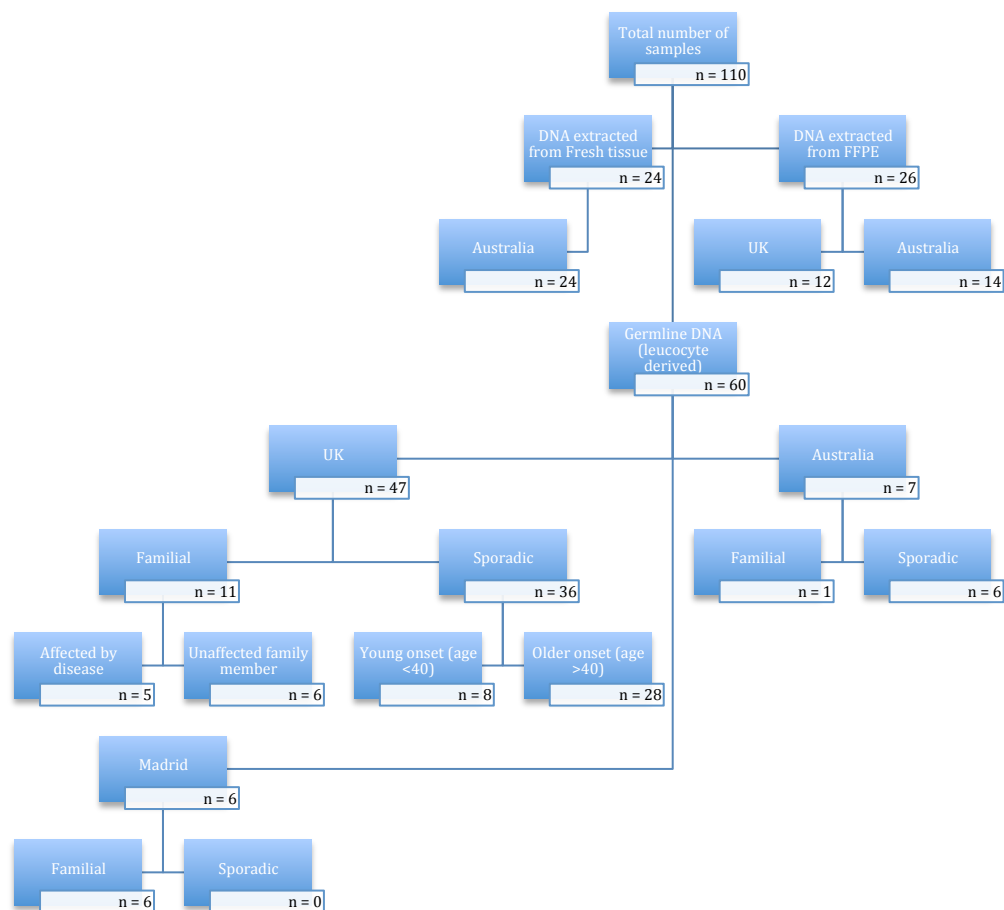


Figure 20. Flow diagram illustrating the number, type and origin of all DNA samples used within this study

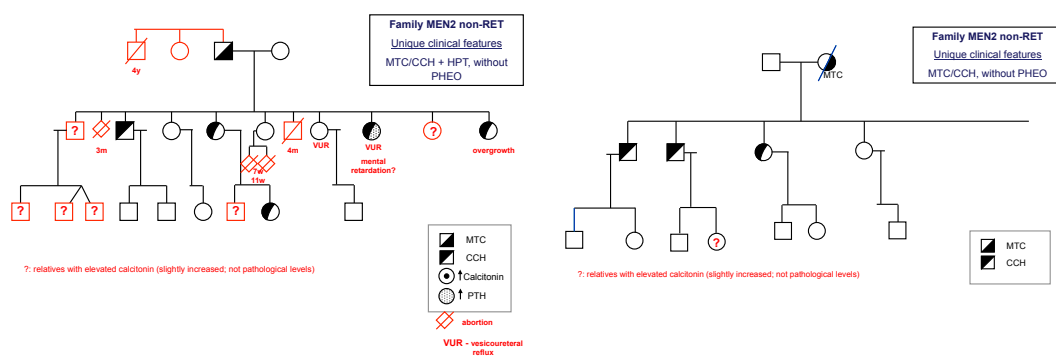


Figure 21. Two further RET negative families with MTC and CCH pedigree obtained from collaboration with Mercedes Robledo, Madrid.

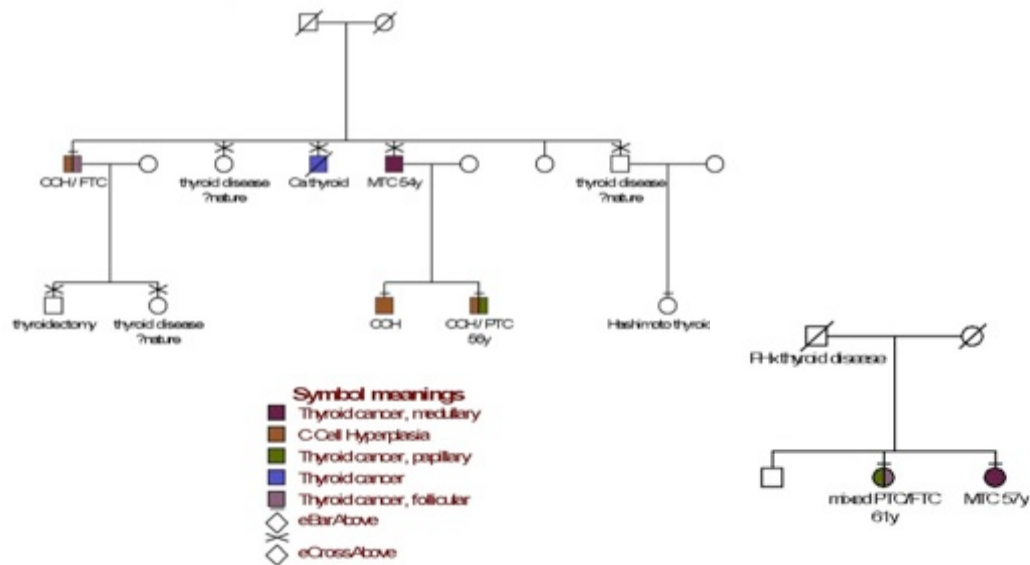


Figure 22. Two further families recruited since the project started with MTC/CCH pedigree.

3.4 Sporadic cohort characteristics

3.4.1 Age and sex characteristics

In the recruited cohort, no significant differences were noted when comparing age and sex characteristics at presentation between patients with RET positive and RET negative disease with a median age of 52 and 60 for RET positive and RET negative disease respectively (Table 21). This comparison is made after excluding those individuals identified through family tracing. Peak age at disease onset was consistent with other series of sporadic MTC as was an equal male to female distribution and although there were more males than females in the RET negative group in the cohort, the differences were not significant and could be explained by relatively small sample size (Figure 25).

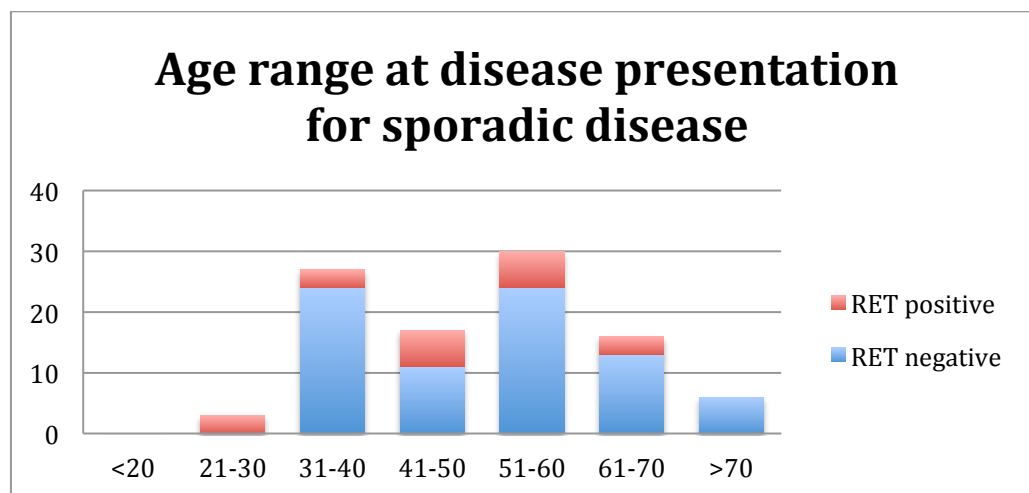


Figure 23. Age range for sporadic and index case RET positive and RET negative familial disease.

Disease presentation	Mean age (range)	Median	SD	p = (paired T-Test)
RET Positive	43.7 (26-52)	52	12.0	-
RET negative	48.6 (31-72)	60	13.4	0.2

Table 21. Age at presentation for germline RET positive and germline RET negative disease

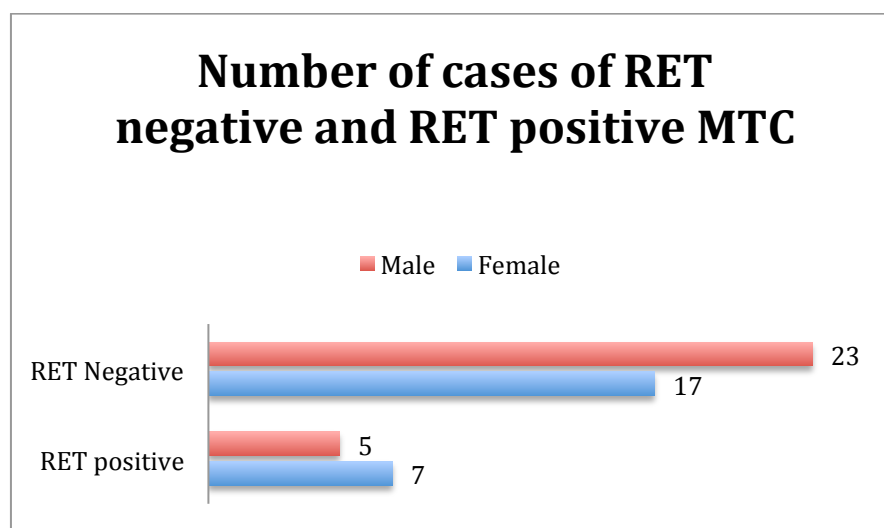


Figure 24. Sex distribution of sporadic RET negative and RET positive disease

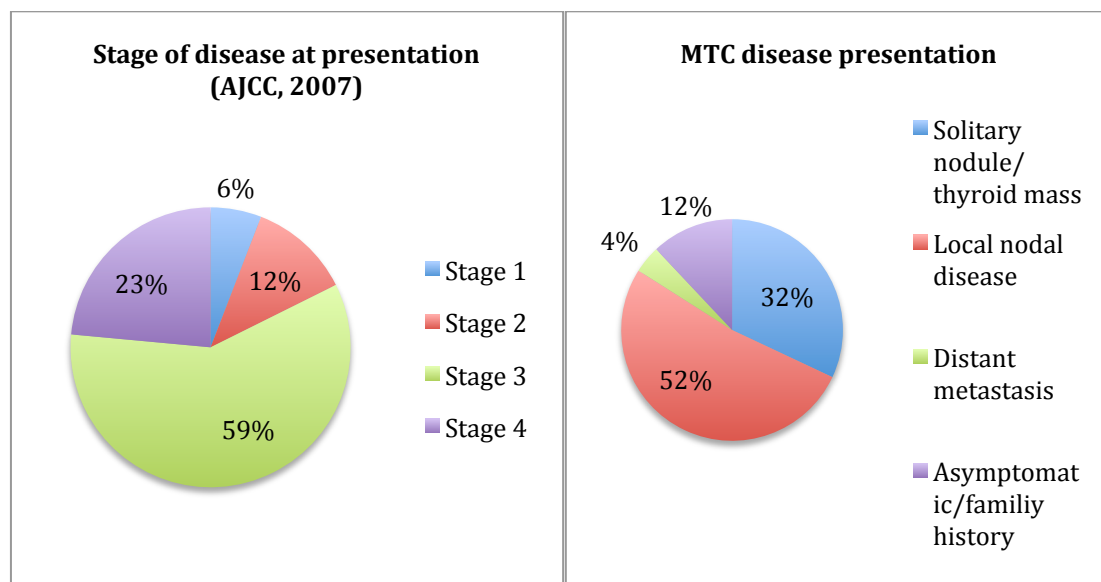


Figure 25. Stage at presentation of sporadic MTC. Figure 26. Clinical presentation of disease.

3.5 Presentation and survival

In terms of disease presentation in sporadic RET negative and RET positive disease presenting as an index case, the vast majority presented with loco-regional disease (nodular thyroid disease or more commonly regional nodal metastasis). There were no differences in disease presentation between RET positive and RET negative disease (data not shown here). Although a relatively small number in this cohort were identified through asymptomatic screening of affected family members, this may reflect the recruitment strategy adopted during this study. Distant metastases were an unusual form of disease presentation although distant metastases in those presenting with loco-regional disease that developed following treatment and follow-up were more common.

Stage I and II disease was an unusual finding in this cohort. The vast majority (82%) of disease in our cohort presented with stage III or IV disease. Follow up with the cohort (time between disease presentation and the time of enrolment in the study) ranged from 2-15 year with a median follow-up period of 8 years (SEM 2.5). At the point of enrolment, 27% were alive with no documented evidence of biochemical or clinical disease. In a further 27% of patients who were alive following treatment, presence or absence of recurrent or residual disease could not be established from available clinical records. The majority of patients with known status (41%) within our cohort have documented evidence of residual or recurrent disease either on clinical or biochemical parameters (Figure 28).

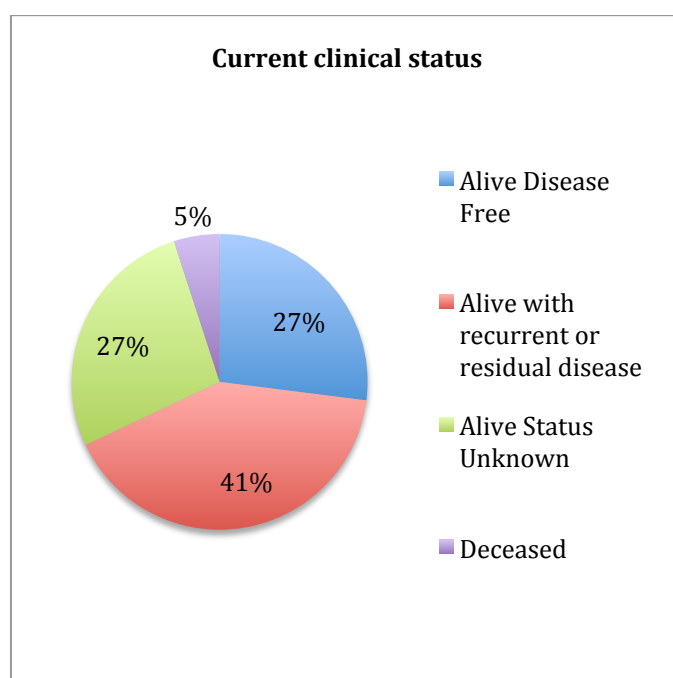


Figure 27. Disease status at follow up following primary treatment.

3.6 Discussion

3.6.1 Recruitment

In line with recommendations for studies of rare diseases, the recruitment strategy, advertising the project and collaborating has significantly increased the number of patients and samples we are able to draw on for the rest of the project (Groft, 2013). This gives us the potential to generate more data when interrogating genes and gain a truer idea of the frequency of any identified mutations. Clearly, at some point, a decision has to be made on a specific mutation or small group of mutations to take forwards for *in vitro* interrogation. Being able to draw on a wider cohort may make this significantly less difficult as the identification of the same mutation or additional novel mutations within the cohort adds weight to genotype/phenotype presumptions. The significance of any finding is related to the individual in which the mutation was identified and the type of mutation and its predicted effect.

3.6.2 Strengths and weaknesses

Although recruitment has been successful and indeed as this represents one of the largest cohorts of patients with MTC currently under investigation, what is somewhat unfortunate is the small numbers of patients in the categories in which we would most be most likely to find mutations, that is, familial disease and disease of young onset. The implication of limited datasets from which to draw direct comparison provides a degree of uncertainty regarding the genes taken forward for further interrogation. Ultimately it may have to be accepted

that this was likely to be a limitation of the study and could fundamentally undermine the ability to prove assertions or fully test hypotheses. However, by maintaining the promotional aspects of the study by collaboration and advertising and presenting preliminary findings, the hope is to build on the current cohort and ameliorate some of the disadvantages of limited cohort size. To this end data have been presented at multiple meetings within the UK and internationally to raise awareness, whilst continuing to collaborate with groups and clinicians to recruit patients. As the project moves forwards and we present and publish findings, the hope is to increase on the numbers presented here and ultimately provide a repository for the identification of multiple mutations important in the development of MTC.

As with novel gene discoveries in more common forms of cancer such as breast, over and above the most prevalent mutations (e.g. BRCA1/2), the remaining burden of inherited disease is made up from numerous mutations in related but ultimately common pathways to those that account for the majority of the disease (Haanpää et al., 2013) (Lee and Ang, 2014). We may have to accept that as such a large burden of inherited disease in MTC is accounted for by RET mutations that all familial cases that do not have either an undescribed RET mutation, novel mutations may be so rare as to be family specific. With this in mind it seems unlikely that the same mutation would be found in multiple unrelated families based entirely in the scarcity of the phenotype. The recruitment of other families to this project however may be a starting point for further investigation in the form of whole exome or whole genome sequencing ultimately working toward a battery of novel mutations in

related pathways from differing families. What is clear is that investigation of rare phenotypes of more common diseases always has the potential to add to the knowledge and understanding of the molecular biology of cancer predisposition and as such has the potential to benefit everyone with that disease.

3.6.3 Cohort characteristics

There are important similarities and differences between our cohort and the wider population of patients with MTC that require further examination in order to validate subsequent results (Roman, Lin and Sosa, 2006). We observe no difference in sex distribution between our cohort of RET negative and RET positive patients. This has implication in gene mutation searching and assessment of the likelihood of mutations causing disease. It would for example be reasonable to assume that mutations on the X and Y chromosomes could be safely eliminated from further interrogation. Equal male and female distribution in cancer is seen across a range of malignancies in differing tissue types. Well-differentiated thyroid cancers however, are significantly more prevalent in women with a number of possible explanations for this, not least the exposure of female thyroid tissue to hormonal factors including oestrogen & the possible balance of oestrogen and androgen receptors (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014c) (Magri et al., 2014). As outlined in the general introduction, C Cells are both embryological and physiologically distinct from the surrounding thyroid tissue. They do however share a common microenvironment so to what extent these factors are important is not known.

The age range, and in particular the median and mean ages of our recruited patients is higher than in some other reported series (Roman, Lin and Sosa, 2006). There are several explanations for this. Firstly, recruitment is a factor as our cohort has inherent bias with a proportion of self-referring patients rather than national data or data extracted directly from locally held databases (UK, 2013). The older group we have identified here may in some part be representative of older patients being more willing to take part in research. Furthermore, as our search strategy has focused on patients with sporadic disease, we have not got significant numbers of patients in younger age ranges, which are likely to skew the data from other large series in which there are significantly more of these individuals. Finally this may be slightly higher than observed in other large series of MTC although again could be partially explained by the recruitment strategy performed to assemble this cohort and the small number of patients identified through contact tracing[6] rather than through clinical signs or symptoms.

3.6.4 Presentation and outcome

our recruitment and cohort differences may go some way to explain the differences we see between the cohort presented here and elsewhere in the literature (Gimm and Dralle, 2001). We certainly have a higher than expected proportion of patients with advanced disease (stage 3 and 4) then presented elsewhere (Hundahl et al., 1998). This could be explained in part by some of the differences outlined within our own cohort in terms of an older group and in a group self-selected by willingness to be involved in the study. It is

possible that those with more advanced disease and, as such less likely to achieve biochemical and clinical cure, have a greater vested interest in being involved in research than those cured at initial surgery due to limited disease. Furthermore, and as previously stated, a bias in our group towards sporadic disease presenting as index cases rather than detection through screening may go some way to explaining this.

Our data on outcome to date is skewed somewhat by missing data on the biochemical and clinical picture currently. The main factor here is that even with copies of clinical letters and follow-up information it is often not clearly stated what the current disease status is. This is an issue for any clinical project where the clinical data has to be collated in a retrospective manner and we accept that this weakens these results somewhat. an attempt will be made to complete this data in the future, but this is beyond the scope of this project. Although there are significant missing data we have no reason to assume that data not being available is for any other reason than a random event. As such, we believe that the overall data would not change significantly, that is to say, that a large proportion of patients with MTC following treatment will remain alive at follow-up but with evidence of either clinically residual disease or with biochemical but clinically unidentifiable residual disease. This is a common finding in the treatment and management of MTC and is a feature of a disease that presents at an advanced stage and has limited treatment options over and above local surgical tissue clearance.

3.6.5 Implications

Overall, a significantly higher rate of advanced disease in MTC when compared to other forms of thyroid cancer is to some extent not surprising when comparing the differing grading systems of thyroid malignancy (Anon, n.d.). The change in the grading system between the two diseases highlights the importance of nodal metastasis in the likelihood of achieving cure. In well-differentiated thyroid cancer, nodal status is of relative unimportance in treatment and although 50% have micro or macroscopic nodal metastases, the difference in overall outcome between those with and without disease is relatively small (Nixon et al., 2014). In part this is due to the effectiveness of ablative radioiodine treatment post-surgery, which effectively controls disease both in the thyroid bed and the nodal compartments (Nam-Goong et al., 2004). In MTC however, evidence of disease spread to nodal groups significantly reduces the likelihood of achieving cure (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014c). As much of this disease is microscopic and not clinically apparent, achieving full clearance of affected tissue surgically, whilst attempting to be conservative is a great challenge (Roman, Lin and Sosa, 2006). The most likely chance of achieving this is at the first operation, and this can be compromised if the disease has not fully been diagnosed. As can be seen by our data presented here, many patients presenting with stage 3 and 4 disease undergo multiple procedures.

Chapter 4.

Clinical and experimental RET testing

4.1 Introduction

4.1.1 Establishing cases

Following recruitment, it was required that both our index family and our wider cohort were genuinely RET negative and therefore suitable for inclusion. As previously discussed, clinical RET testing is offered to all patients with a diagnosis of MTC with familial testing and further cascade testing as appropriate (if a mutation is confirmed) (Perros, Boelaert, Colley, Evans, Rhodri M Evans, et al., 2014c). Clinical RET mutation testing at UHB laboratories currently consists of capillary sequencing (Sanger) of exons 10, 11, 13-16 as it is within these coding regions that the majority (95%) of disease causing mutations will be present (Mulligan, 2014). The exons tested and the sequence of testing differs between labs (Romei et al., 2011). Our methodological approach is based on the assumption that alteration in gene expression and or gene mutations other than the RET are implicated in the pathogenesis of MTC. Ensuring RET negative familial and sporadic cases are genuinely RET negative is therefore of vital importance if that hypothesis is to be tested. In a similar study involving WES in a Chinese family, the WES result actually identified a novel RET mutation rather than a novel gene mutation (Qi et al., 2011).

As 5% of RET mutations (disease causing or potentially disease causing) will be outside of the exonic regions screened by clinical testing, it is prudent to screen the remaining exons for mutations and to ensure genuine RET negative status. Although primarily capillary sequencing has been completed

here, in cases that later went on to have WES, a secondary check was available. Although not the only method available, capillary sequencing is considered to be the gold standard for mutational analysis (Margraf et al., 2006), (Bugalho, Domingues and Sobrinho, 2002). Although as the reliability and speed of next generation sequencing improves, this may well take over as a diagnostic screen (Strom et al., 2014).

Sequencing as described by Sanger, involves amplification of short segments of targeted DNA by thermal cycling and using the principles of polymerase chain reaction (PCR) amplification (Sanger, Nicklen and Coulson, 1977). Genomic or somatic DNA extracted from blood or tumour cells is purified prior to melting at a specific temperature which leads to the two strands separating (Shendure et al., 2004). Specific primers (oligonucleotides designed to bind to the '5 and 3' end of the target sequence) can then anneal to the DNA and the sequence extended using a polymerase on the presence of an optimised mix of deoxynucleotides (dNTPs). Thermal cycling through phases of melting, annealing and extending, ultimately lead to exponential increases in the quantity of the amplicon (the small segment of DNA that is being amplified). The amplicon can then be purified, removing excess dNTPs and template DNA prior to use in the sequencing reaction. With regard to RET this practically involves primer pairs for each of the 20 exons, with multiple primers for amplicons larger than 400bp.

4.1.2 PCR amplification

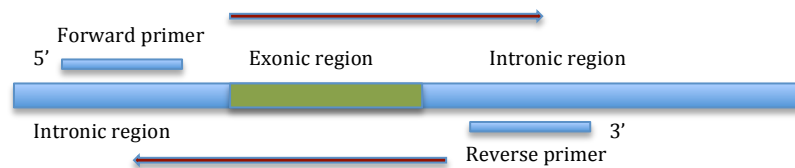


Figure 28.

Intronic primer design for PCR amplification of a single exonic region <400bp

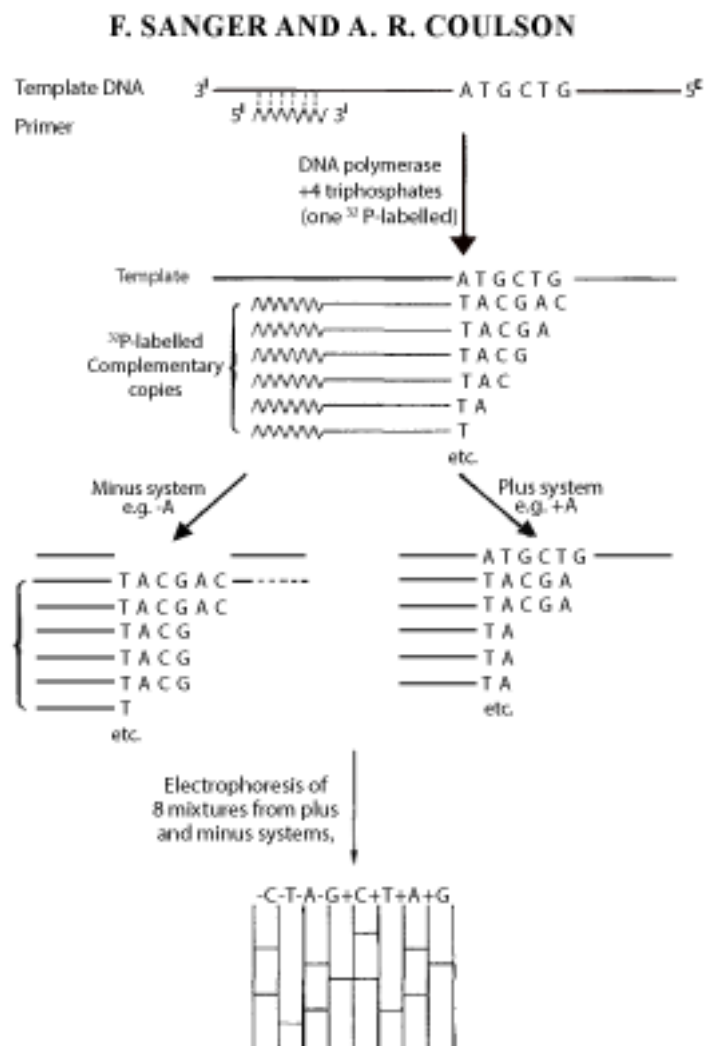


Figure 29. DNA sequence recognition by using oligonucleotide primers with extension by DNA polymerase (Sanger, Nicklen and Coulson, 1977)

4.2 Methods

4.2.1 Primer design

Primers were designed as previously described.

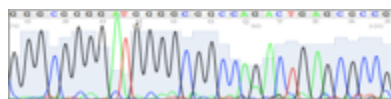
Oligonucleotide	Sequence	Redesign needed
RET Exon 1 Forwards	GCGCTTACCTCGCTTCAGT	-
RET Exon 1 Reverse	ACAGAAAGGCGCTTCTGAAC	-
RET Exon 2 Forwards	TTCTACCATCCCTCACTCA	TTTTTGCCTTGAAGAAGCCTTA
RET Exon 2 Reverse	GTGTCAGCGGCTGTGATAAG	-
RET Exon 3 Forwards	CCCCACAGACCTGACTTCTC	-
RET Exon 3 Reverse	GAGCAAGACCAGCAGTAGCA	-
RET Exon 4 Forwards	CAAAC TGCAAAC TCGTGCTC	CAAAC TGCAAAC TCGTGCTC
RET Exon 4 Reverse	ACACTGGGTAGACCACAGCA	GACCGAGAAACGAACTGTGG
RET Exon 5 Forwards	ACTGACCAACGCCCTCTG	GGACGTGCAGCATTCTAAGG
RET Exon 5 Reverse	AAGAGCGAGCACCTCATTTT	-
RET Exon 6 Forwards	AGAGCAGCTTGGTGGTCATT	ACATGAGGAAGCAGCCAGAG
RET Exon 6 Reverse	CCCCAGACAGGCAATAGGTA	GTCACCTGCCTCCCTGTG
RET Exon 7 Forwards	AGCTGCCTGGCTAAGGTGT	TACCCTCAGGCCATTACAGG
RET Exon 7 Reverse	AGGCCCAGGCTCCAGAAG	GCTTTTCTCAAAGGGCAGGA
RET Exon 8 Forwards	CCTGTGACCCTGCTTGCT	-
RET Exon 8 Reverse	AGACCTGGAGCAGGGAGAG	-
RET Exon 9 Forwards	GATCTGCCTAGGAGGTGGTG	GATCTGCCTAGGAGGTGGTG
RET Exon 9 Reverse	GTTCCCATGCCCTGATTAAG	TCTCCTACCTGTTCCCATG
RET Exon 12 Forwards	CCCCTGTCATCCTCACACTT	-
RET Exon 12 Reverse	GGCAGGTACCTTTCAGCATC	-
RET Exon 17 Forwards	AGCCACTCACTGGTCCTTTC	CTCTGTGAGGGCCAGGTG
RET Exon 17 Reverse	GGAATGCACACAGATGTCC	-
RET Exon 18 Forwards	GGCTGTCCTTCTGAGACCTG	-
RET Exon 18 Reverse	CAAGCCACTTTCAGCTTGTG	-
RET Exon 19 Forwards	CAAGCCACTTTCAGCTTGTG	TAGTTGTGGCACATGGCTTG
RET Exon 19 Reverse	CAAGCCACTTTCAGCTTGTG	GATAGTGCAAAGGGGACAGC
RET Exon 20 Forwards	TTGCCAAGGCCTTACTGTCT	-
RET Exon 20 Reverse	TCTTCCCCTTGTGAGTCCAT	GAAAGGGCCGGTAGACTTTC

Table 22. Intronic RET oligonucleotides to capture sequences for exonic regions not covered in conventional clinical testing.

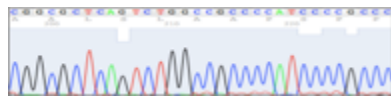
4.3 Results

Germline DNA was initially available for 24 patients with sporadic MTC for sequencing analysis. All patients had had laboratory clinical testing of RET exons 10, 11, 13-16 with no RET mutation identified by Sanger sequencing. After manipulation of cycling parameters and primer pairs all remaining exons were sequenced and screened for mutations. Figure 32 shows representative samples from each exonic region. No novel mutations were found in these patients (0/24). Single nucleotide polymorphisms (SNPs) were found in exon 5 and exon 7 with frequency within the sample of 17% (5/24) and 33% (8/24) for each SNP respectively. Mapping and characteristics of these polymorphisms can be seen in Figure 34.

4.3.1 Sequencing was performed in all exons not tested routinely by the clinical laboratory



Exon 1 Forward



Exon 1 Reverse



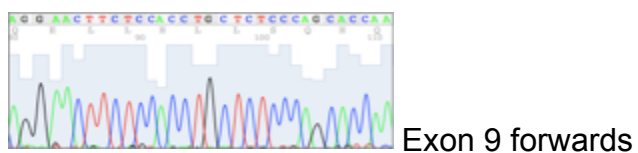
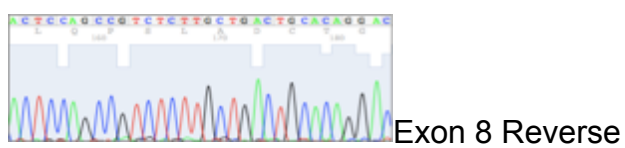
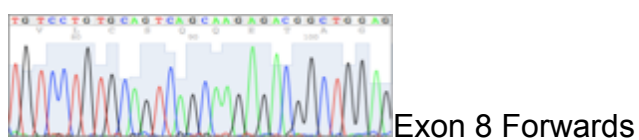
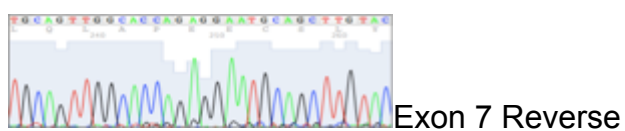
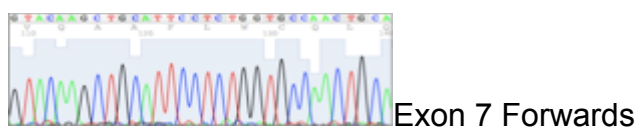
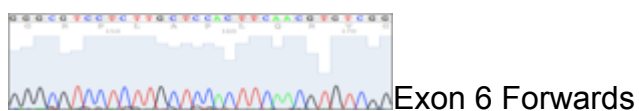
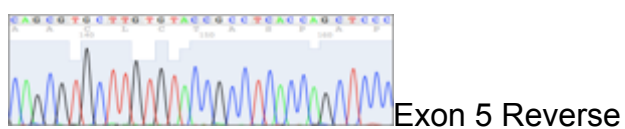
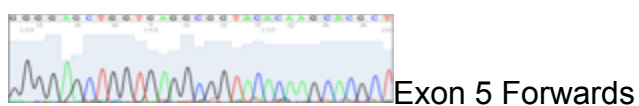
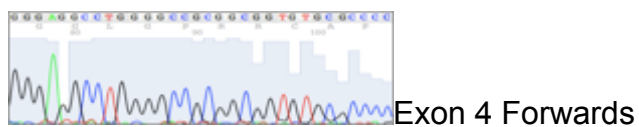
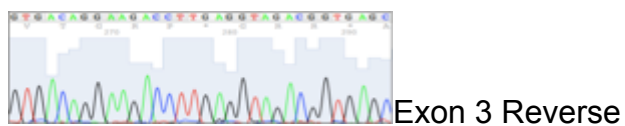
Exon 2 Forward



Exon 2 Reverse



Exon 3 Forwards



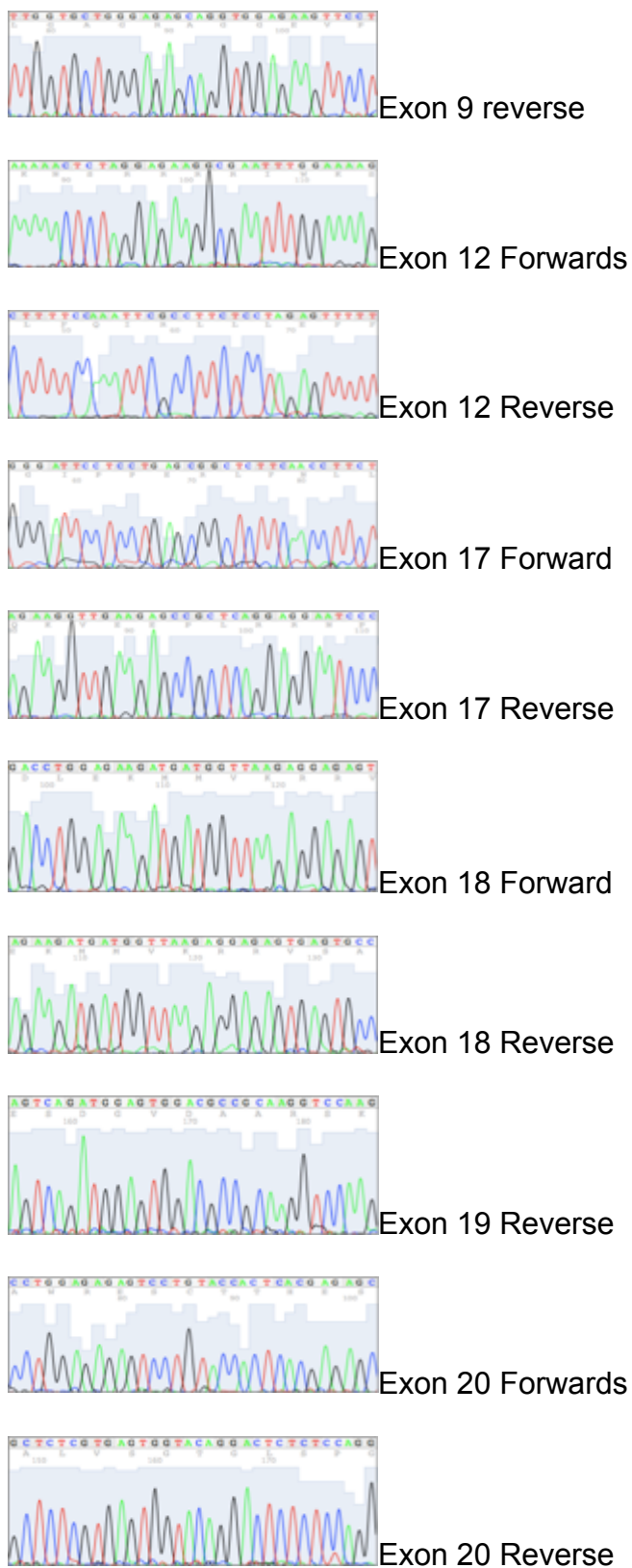


Figure 30. Representative sections of chromatograms for each exon not routinely tested clinically

4.3.2 Possible mutations were checked on the reverse strand before logging as SNPs or mutations.

Where reverse strand verification was not obtained the experiment was repeated. Figure 32 shows a possible mutation in the forward trace with T to C heterozygous change. The reverse trace from the same patient's DNA can be seen below which shown normal signal at that position.

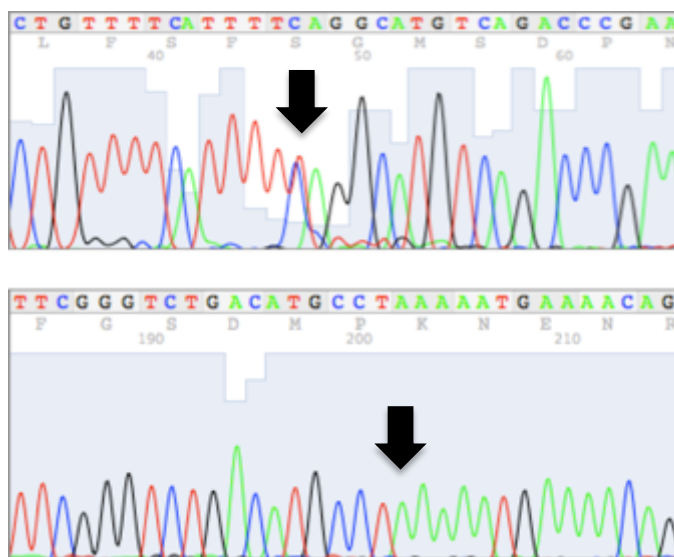
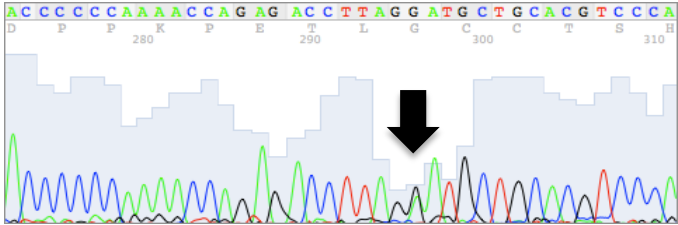


Figure 31. An apparent mutation on RET exon 20.

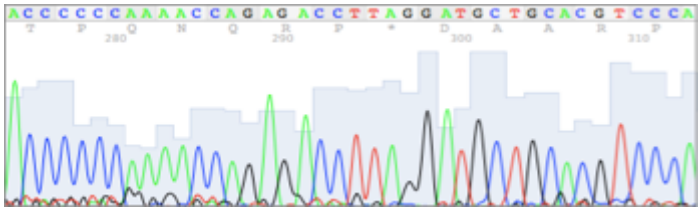
4.3.3 Multiple SNPs were identified through screening of the untested exons

The most commonly identified SNPs were in exon 5 and exon 7. On exon 5, reverse strand is not readable at this position of this SNP due to its proximity to the primer binding site, but as the SNP is well described, the experiment was not repeated. Figure 33 shows the mutation on the forward strand with two overlying peaks. The corresponding normal trace the peak size of the G

at the position of the mutation is halved compared to normal, again indicating this is a heterozygous mutation. Figure 34 shows the SNP on exon 7 found in 8/24 patients with apparently sporadic disease.

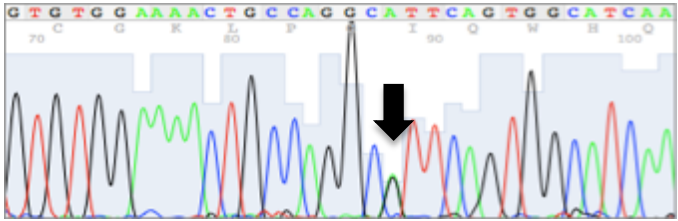


Exon 5 Forward strand

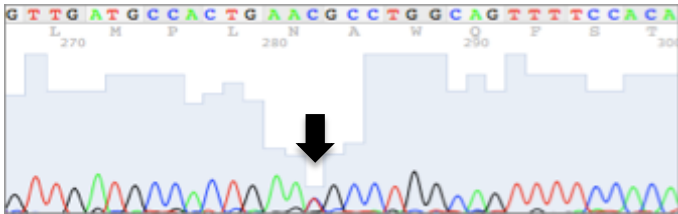


Exon 5 Forward strand normal

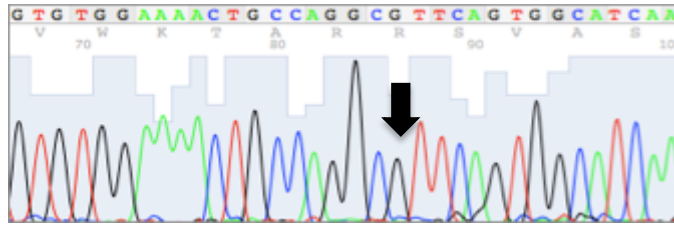
Figure 32. Patients within the sample cohort carry this G to A mutation in RET exon 5.



Exon 7 Forwards



Exon 7 Reverse



Exon 7 Normal forwards

Figure 33. RET Exon 7. 8 patients from the cohort of 24 have this heterozygous G to A mutation in exon 7.

4.4 Discussion

4.4.1 Mutation of the RET gene outside of the exons tested clinically are rare in sporadic MTC

An important first end point to this project is the verification of genuine RET negative status in our both our index family and in our initial cohort of patients with apparently sporadic disease. We found no novel, non-synonymous changes in the RET gene. Two common SNPs in the RET gene were the most common alteration identified, but neither differed significantly in frequency from other analyses SNP databases (Karolchik et al., 2008). This is in no way a surprising finding, although important to establish as a point of principle for further investigation. As reported, 95% of all RET mutations that are established as disease-causing occur in exons 10, 11, 13-16 (Ponder, 1999). RET mutations outside of these exonic regions have been reported as disease causing in MEN 2a (exon 8) (Bethanis et al., 2007). Where functional work has not established the molecular basis of disease, however, there is some dispute as to the disease causing effects of some rare mutations of RET (Eng and Mulligan, 1997). Further work is required before some of the identified mutations are routinely screened for clinically (Figlioli et al., 2013).

4.4.2 The index family are RET negative confirmed by Sanger sequencing and therefore suitable for interrogation using WES

With next generation sequencing still in its infancy, Sanger sequencing is still considered the gold standard for mutation identification and confirmation (Bugalho, Domingues and Sobrinho, 2002). As seen elsewhere in the

literature, families with MTC/CCH pedigrees that are assumed to be RET negative have subsequently been shown to harbour novel mutations (Qi et al., 2011). The results presented here confirm that this is not the case and as such the index family represents an ideal start point for identification of novel MTC predisposing mutations.

4.4.3 Based on these data, further RET testing within our wider cohort has not been performed

As the focus of the project has moved on toward the examination of WES results a decision was made to not further interrogate samples from patients subsequently recruited to the project following the results for this initial cohort of 24. The size of the RET gene makes the interrogation by Sanger sequencing both time consuming and expensive in terms of consumables and as the yield in terms of potential new mutations is likely to be small. It is possible that there are RET mutations in our wider sample that we will miss by taking this approach however, based on the data presented here, the numbers are likely to be low and as such not significantly bias our results. The major consequence of missing large numbers of mutations would be to dilute the true frequency of other identifiable candidate mutations. As ultimately, novel candidate are likely to occur with low frequency, this is a justifiable approach.

Chapter 5.

Whole Exome sequencing and analysis

5.1 Introduction

5.1.1 Whole Genome and Whole Exome Sequencing (WES)

WES is a form of ‘next generation’ sequencing whereby an attempt is made to capture all of the coding regions (exons) of the genome (Ng et al., 2009). The exome represents 1% of the genome but is thought to harbour more than 85% of disease causing mutations (Mamanova et al., 2010). In humans, this region represents over 20,000 consensus coding regions accounting for over 18,000 genes (Kim D. Pruitt et al., 2009). Using a method of exome examination is therefore a pragmatic solution to disease causing mutational analysis (Robinson, Krawitz and Mundlos, 2011). With respect to this project, WES has the advantage of being able to accurately unpick genotype/phenotype relationships between highly penetrant genes (as expected in the index family), where multiple samples are available and where the likely causative mutations are exonic, non-synonymous, frameshift or canonical splice variations (Biesecker, Shianna and Mullikin, 2011a). This stands in contrast to whole genome sequencing (WGS) where interrogation of small numbers of individuals with low penetrance and complex disease genotype phenotype relationships.

The key to WES success is ‘targeted capture’ of the exome and various commercial applications are available employing similar technology. The technologies are similar in that they essentially employ nucleic acid probes and primers designed to hybridise with fragmented genomic DNA (Summerer, 2009). Differences between capture platforms do exist however, most notably

in the type and character of capture probes used and in solid and liquid phase capture techniques (Summerer, 2009). The differences in methodology give commercial platforms different characteristics and will inevitably create variation between samples, both in terms of the depth and extent of the proposed exome coverage.

Exome 'definition' differs somewhat between databases and in some cases and there is a lack of clear consensus of what exactly constitutes the coding region of a gene. Although coding of the human genome is complete, evolution of information regarding start and stop codon position and splicing variants is ongoing and may lead to differences between datasets (Kim D Pruitt et al., 2009). Work by the consensus group bringing together data from commonly used data sets such as National Centre for Biotechnology (NCBI), the Ensembl Genome Browser and University of California Santa Cruz (UCSC), has provided a point at which agreement can be made on key genomic coding properties (Birney et al., 2004), (Karolchik et al., 2008). To some extent these current next generation platforms and the results generated from them will be dictated by the exome definition used, the database or consensus and as such the specific probes used and the subsequent sequence capture. Some of these issues will likely diminish with time as an increasing amount of next generation sequencing takes place more data covering non-consensus regions is analysed.

WES technology has recently been used in the investigation of MEN 2A where novel germline RET mutations were discovered (Qi et al., 2011).

Exome sequencing has also been used to delineate mutation in other diseases including identifying candidate genes conferring genetic susceptibility to pheochromocytoma (Comino-Méndez et al., 2011). As such it makes WES an appropriate strategy for genetic investigation of both uni- and multifactorial disease (Michael J Bamshad et al., 2011), (He et al., 2011). Although it is accepted that whole genome sequencing (WGS) will most likely replace WES, the costs are currently prohibitive for most research projects. Further considerations for employing WES over WGS are managing the quantity of bioinformatics data produced and the time in which modern sequence analysers can process the results (Michael J Bamshad et al., 2011).

As well a pragmatic approach based on the likelihood of mutation discovery, WES has a significant advantage in terms of both scale and cost with regard to traditional sequencing techniques with an estimate of a 10 fold cost reduction and significantly higher genome coverage. The current estimated costs for WGS and WES at the time of writing are 20k and £800 respectively. The advantage of WES over WGS is primarily based on cost but also on the current abilities to manage the quantity of data and the likely need for bioinformatics input and associated costs produced by WGS. Current estimates of cost for WGS is in the order of 20 times that of WES due to access to analysers but also in the time WGS takes on each machine and the implications for flow through when processing multiple samples (Biesecker, Shianna and Mullikin, 2011a).

Next generation sequencing has led to a significant change in the scope and scale of genomics with significant expansion expected in the area (Robinson, Krawitz and Mundlos, 2011). Increasing availability and decreasing costs, offers a genuine shift in research paradigm from hypothesis testing to hypothesis generating. rather than developing a hypothesis based on clinical or biological behaviour of a cancer and developing an interrogation strategy to detect potential imbalances in pathways that could lead to cancer predisposition, all pathways, known and unknown can be interrogated simultaneously. Clearly, the key advantage in this situation is to provide evidence or linkage within and between pathways not previously considered.

Designing an effective strategy that is inclusive of the type of mutations that could be disease causing without inappropriately eliminating potentially interesting findings will always be a challenge when managing such large quantities of data. Figure 36 highlights some of the data filtration strategies that can be employed based on possible clinical sampling scenarios. In strategy 1, the assumption of novelty between generations is applied. In strategy 2, multiple, non-related individuals are sequenced and mutation data cross-referenced to search for similar mutations. In strategy 3, rare phenotypes of more common disease are used as target points for identification of genetic clustering. In strategy 4. assumptions based on the inheritance pattern of a disease, in this case, autosomal dominant allowrecessive mutations to be screened out (Michael J Bamshad et al., 2011)

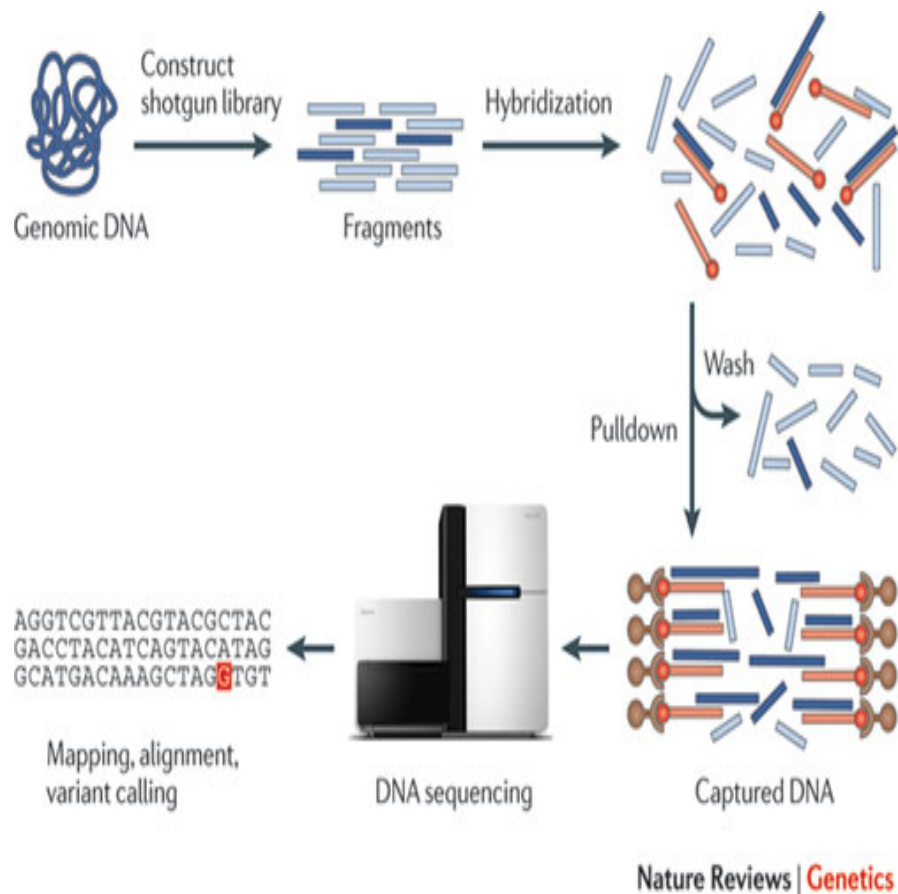
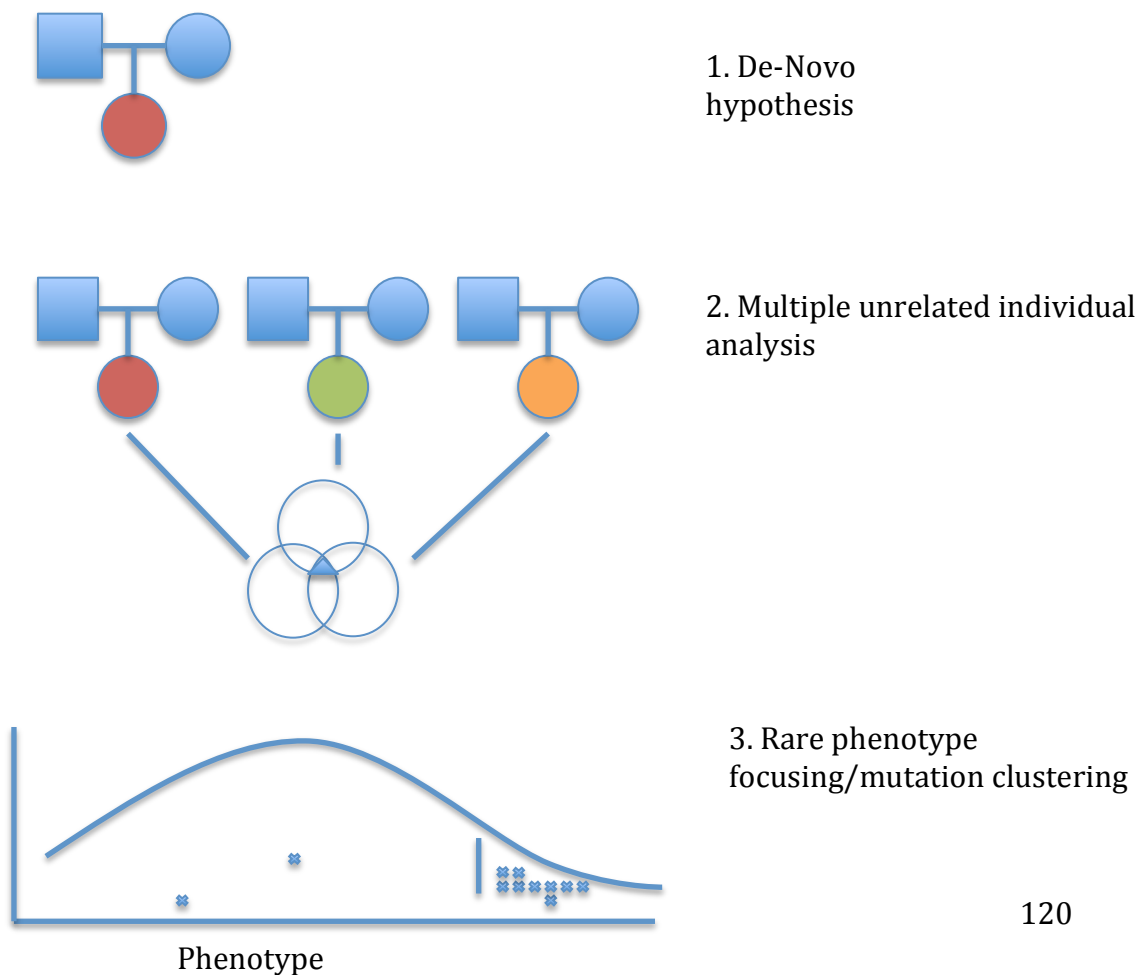
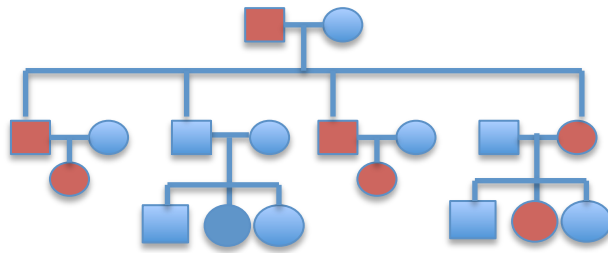


Figure 34. Schematic representation of WES methodology from extracted genomic DNA (Michael J. Bamshad et al., 2011).





4. Multiple affected members of the same family. In this situation choosing comparators most distantly affected is likely to yield the best results.

Figure 35. Strategies for identification of a targeted list of mutations based on disease specific, inheritance and novelty assumptions. ((adapted from (Michael J Bamshad et al., 2011))

5.2 Materials and Methods

5.2.1 Patient selection for Whole exome sequencing (WES)

WES was initially performed on two members of the index CCH/MTC positive family (S0288 and S0289). A further two samples added to the analysis to aid in bioinformatics approach (the parents of the proband: S0540 and S0541 Figure 38). Sequencing and data filtration was performed as previously described.

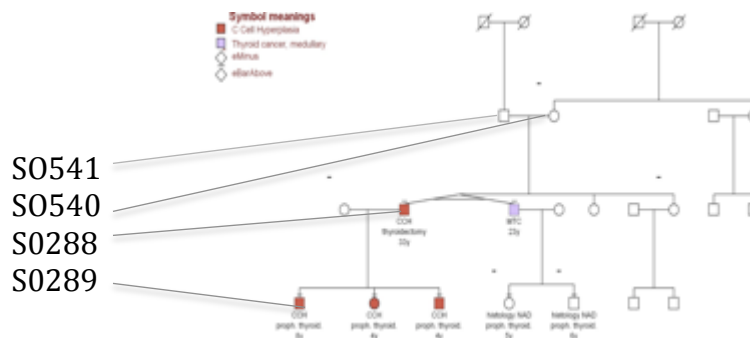


Figure 36. Exome sequencing election within the index family

5.3 Results

5.3.1 Whole Exome Sequencing (WES)

Exome sequencing was successfully completed in all four selected individuals. Table 23 shows the average base read depth as well as the proportion of the exome covered at greater than 20 x read depth.

Sample	Mean base coverage (X)	% of exome covered >20X
S0288	105.18	89.29
S0289	62.23	82.46
S0540	89.04	85.69
S0541	70.87	83.89

Table 23. Exome coverage based on capture techniques including average number of per base capture and percentage of bases captured >20X in each sample.

5.3.2 Whole exome sequencing: selecting candidate genes

Type and proportion of mutation for each dataset are shown in Table 24 and 25. Search strategies using the described bioinformatics assumptions are shown in Figure 38 (de-novomutation assumption) and Figure 39(rejecting the de-novo assumption). In the de-novo approach, all four datasets were combined and 13 novel mutations were identified between the proband and offspring and the parents of the proband. All novel alterations were non-synonymous. Four of the 13 novel mutations were predicted to be damaging on *in-silico* analysis (SIFT and Polyphen2). Table 26 shows the 13 novel mutations with further analysis of the 4 predicted damaging mutations shown in Table 27.

In the second search strategy, where the de-novo assumption was rejected (the parents of the proband were removed from the analysis), 382 novel mutations were identified. The initial focus was on those mutations most likely to alter protein structure. There were 36 identifiable frameshift and non-frameshift insertions and deletions, 7 of which were common to both samples and 2 of which were novel frameshifts. There were 346 novel, non-synonymous changes of which 28 were common to both datasets (Figure 39). No RET mutations were identified in any of the datasets.

Exome Sequence	Total number of mutations	Novel Change	Exonic Mutation	Splicing mutation
S0540	23 736	64	21 086	2650
S0541	23 757	82	21 050	2707
S0289	23 509	261	19 077	4432
S0288	24 342	283	19 756	4586
Total	95 344	-	-	-

Table 24. Total number of mutation within each data set prior to data filtration strategies (It should be noted that the data sets S0540/S0541 were performed at a later date than S0288 and S0289 and as such novel changes highlighted from these data sets were not subsequently recorded as novel in preceding analysis explaining the disparity).

Exome Sequence	Synonymous	Non-synonymous	Unknown	In-frame insertions and deletion	Frame shift insertions and deletions	Stop loss/gain
S0540	10 786	9686	33	286	201	46/83
S0541	10 744	9671	-	307	196	26/87
S0289	10 677	9658	-	240	196	38/74
S0288	10 922	10 068	-	261	208	37/83

Table 25. Frequency of mutation in each dataset

5.3.3 Data filtration strategies and candidate analysis

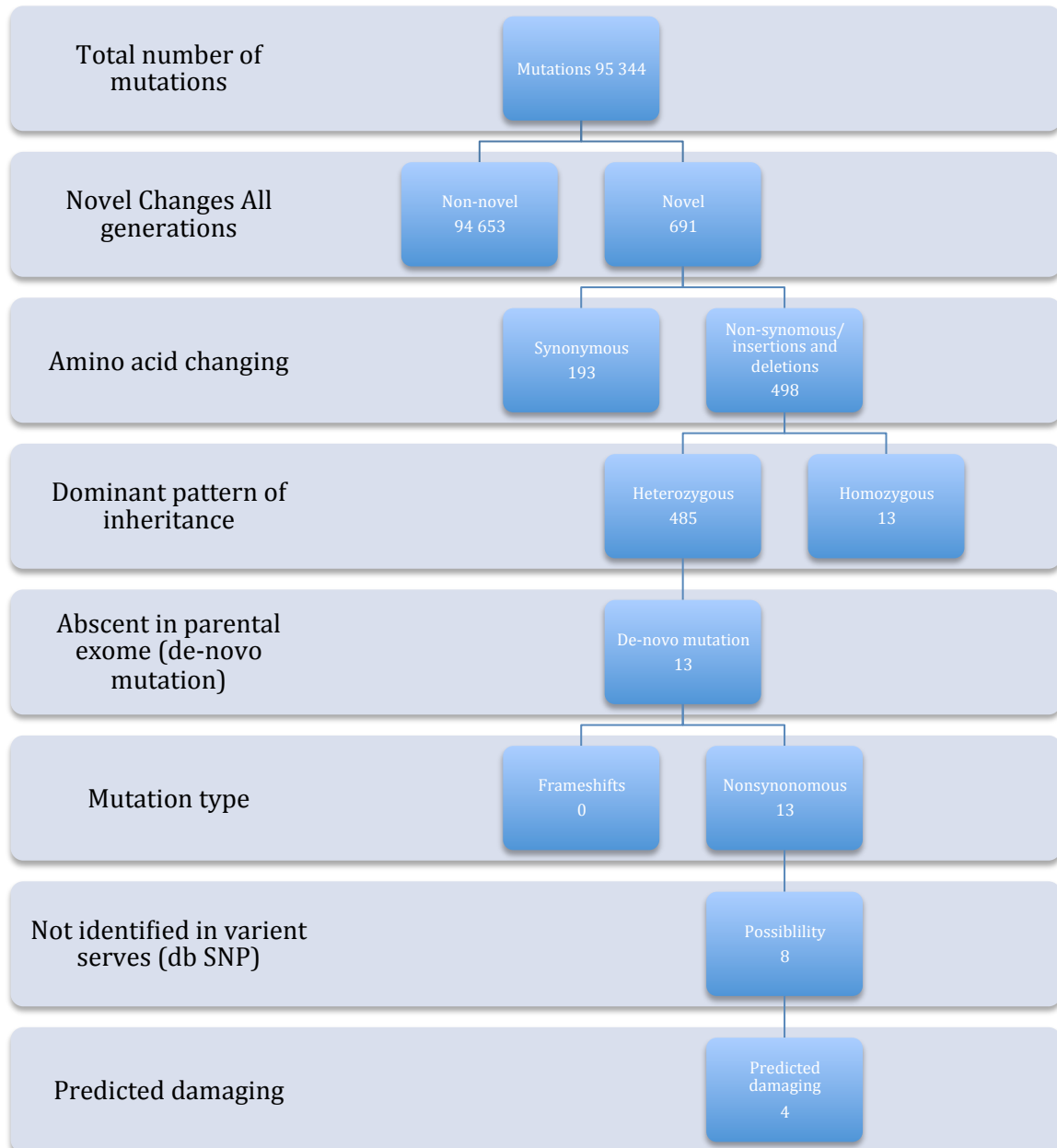


Figure 37 Data filtration using the de-novo assumption. The de-novo approach shown here uses the assumption that the disease causing mutation is novel in the affected proband and twin and as such, exome data from the parents can be used to filter out non-relevant mutations. Total number of mutations screened is the combination of all 4 datasets within the family. The de-novo approach employed here identifies 13 novel mutations between generations all of which are non-synonymous in nature and of which 4 are predicted damaging by in-silico analysis. The 13 novel candidate de-novo mutations are further analysed in Tables 26 and 27.

Gene name	Chr	Mutation type	Identifier (NM)	Variant server check	Polyphen/Sift	Candidate
ZMYND8	chr20	Non-synonymous SNV	NM_012408:exon19:c.C2975T:p.A992V	Not in variant server	?	*
UPF0639	chr14	Non-synonymous SNV	UPF0639:NM_001161498:exon11:c.C1096T:p.R366W,	No reported SNP	Probably damaging	**
RBM4B	chr11	Non-synonymous SNV	RBM4B:NM_031492:exon2:c.A335G:p.D112G,	rs147572851		-
PKD1	chr16	Non-synonymous SNV	PKD1:NM_000296:exon15:c.C6001T:p.R2001W	Not in variant server	46exons: exon 25 huge	*
NDUF B1	chr14	Non-synonymous SNV	NDUF B1:NM_004545:exon2:c.G257A:p.R86Q	rs145473110		-
MUC4	chr3	Non-synonymous SNV	MUC4:NM_018406:exon2:c.C6077T:p.S2026F	Not in variant server	Probably damaging	**
MED29	chr19	Non-synonymous SNV	MED29:NM_017592:exon1:c.G11A:p.S4N,	Not in variant server	Probably benign	-
CYP2F1	chr19	Non-synonymous SNV	CYP2F1:NM_000774:exon6:c.T790C:p.F264L,	Not in variant server	Probably benign	-
CRIPAK	chr4	Non-synonymous SNV	CRIPAK:NM_175918:exon1:c.G749A:p.R250H	rs143316662	-	-
C11orf59	chr11	Non-synonymous SNV	C11orf59:NM_017907:exon2:c.G109A:p.E37K	rs144101476	-	-
BCL9	chr1	splicing	.	Not splicing		-
ATP13A3	chr3	Non-synonymous SNV	ATP13A3:NM_024524:exon28:c.G3070A:p.G1024S	Not in variant server	Probably benign	-
APPL2	chr12	Non-synonymous SNV	APPL2:NM_018171:exon8:c.C560T:p.A187V	rs140436242	-	-

Table 26. Candidate genes mutational analysis using a de-novo assumption approach.

Gene	Biological preservation	Known interactions	Candidate score
ZMYND8	Yes	Gene encoding protein kinase C binding protein. Antigen identified in T cell lymphoma. No phenotypic changes in heterozygous mouse. Homozygous mice do not survive beyond weaning (Gardin and White, 2011).	**
UPF0639	Yes	Pleckstrin homology domain containing, family D. Phospholipid binding properties. (PLEKHD1).	*
PKD1	Yes	Needed for regulation of renal tubular development and ciliated flow. Major gene implicated in the development of polycystic kidney disease (Kurbegovic and Trudel, 2013).	*
MUC4	Yes	Role in production of mucin for maintenance of epithelial surfaces. May play a role in tumour progression. MUC4-ERBB2-ERBB3-NRG1 complex has been shown to down-regulation of CDKN1B, resulting in repression of apoptosis and stimulation of tumour growth. Implicated in multiple tumours including glioblastoma (Li et al., 2014)	**

Table 27. Biological evaluation of candidates identified using the de-novo approach

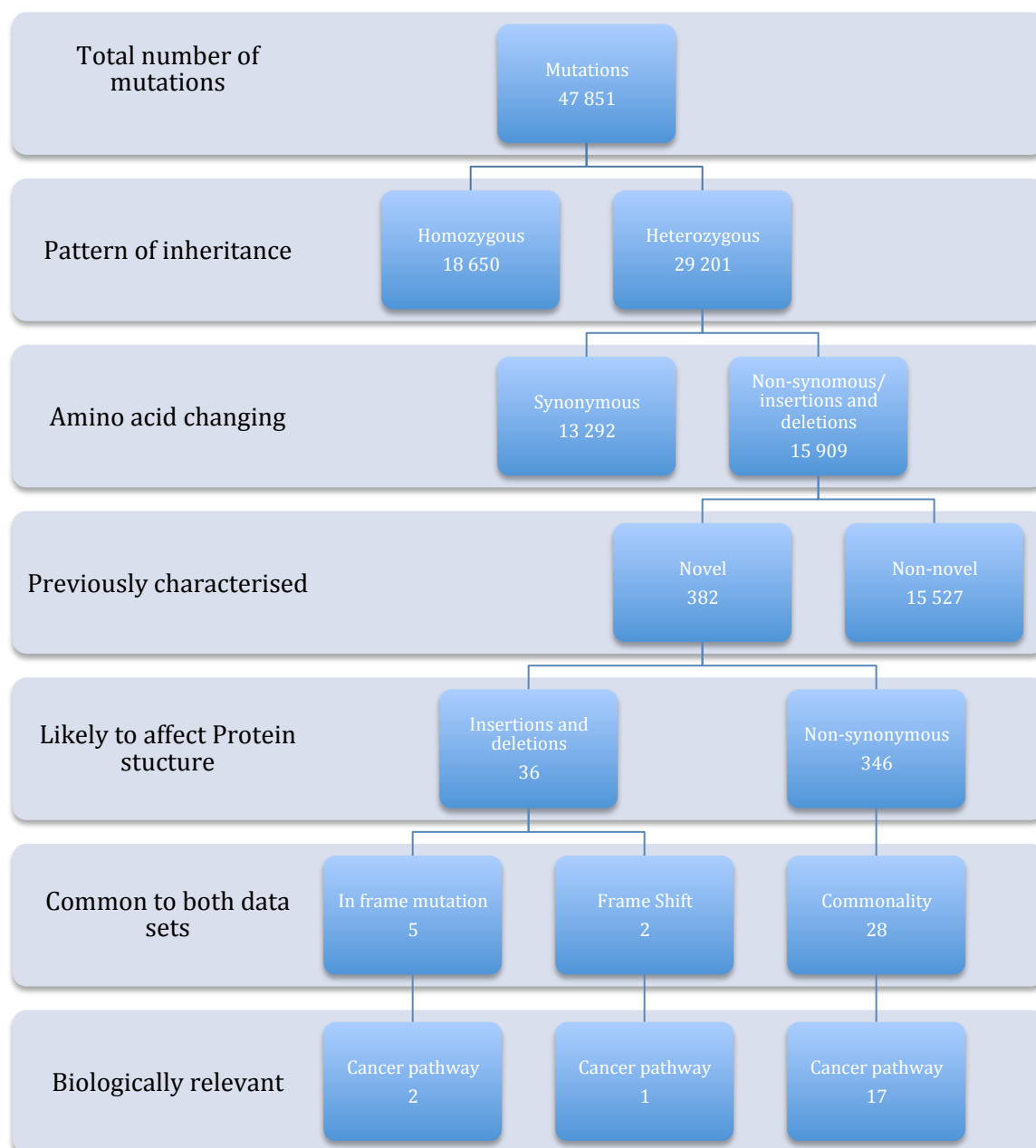


Figure 38. Data filtration strategy excluding the de-novo paradigm. In this approach the assumption is made that the disease causing mutation has not occurred as a novel change between generations. The data presented here represent the amalgamation of datasets in the proband's twin and affected offspring (parental datasets excluded as a filter). Using this approach, 382 novel potential mutations were identified of which 35 were common to both datasets. Alterations in biologically relevant pathways were analysed in further detail and shown in Table 28. Candidate mutations warranting further analysis based on candidate scoring are shown in Table 29.

Gene name	Chr	Mutation type	Identifier (NM)	Variant server check	Polyphen/SIFT	Candidate
BCL9	1	splicing	-	Not in variant server	Not splicing	-
CARD11	7	Non-synonymous	CARD11:NM_032415:exon21:c.T2780C:p.L927P	rs201921426	Yes	-
MED17	11	Non-synonymous	MED17:NM_004268:exon6:c.G938A:p.R13Q	COSM690935 variant server	SIFT tolerated/possibly damaging	*
NUP98	14	Non-synonymous	NUP98:NM_139132:exon22:c.C2983T:p.R995C	Not in variant server	Probably benign	-
RBL1	20	Non-synonymous	RBL1:NM_002895:exon17:c.A2387G:p.Y796C	rs148714635	Damaging	-
TTC23	15	Non-synonymous	TTC23:NM_001040660:exon7:c.C715A:p.Q239K,	Not in variant server	Probably benign	-
C8orf42		Non-synonymous	C8orf42:NM_175075:exon3:c.C377A:p.T126N	Not in variant server	possible	-
FOLR3	11	Non-synonymous	FOLR3:NM_000804:exon3:c.G314A:p.C105Y	COSM690805	Damaging	*
JMJD6	17	Non-synonymous	JMJD6:NM_015167:exon1:c.G44C:p.S15T	rs201990497	Benign/deleterious	-
EXD3	9	Non-synonymous	EXD3:NM_017820:exon16:c.C1713A:p.A571A,	rs372293078	-	-
FAM166B	9	Non-synonymous	FAM166B:NM_001164310:exon5:c.G733C:p.V245L,	rs371340502	-	-
KLHDC4	16	Non-synonymous	KLHDC4:NM_017566:exon6:c.A559G:p.R187G, KLHDC4:NM_001184854:exon4:c.A388G:p.R130G,	Not in variant server	Benign	-
LOC81691	16	Non-synonymous	LOC81691:NM_001199053:exon3:c.G213T:p.K71N, LOC81691:NM_001144924:exon3:c.G213T:p.K71N, LOC81691:NM_030941:exon3:c.G213T:p.K71N,	Not in variant server	Benign	-
MICALCL	11	Non-synonymous	MICALCL:NM_032867:exon3:c.C994G:p.Q332E,	Not in variant server	possible	-
NCKIPSD	3	Non-synonymous	NCKIPSD:NM_184231:exon9:c.C1490G:p.S497C, NCKIPSD:NM_016453:exon9:c.C1511G:p.S504C,	Not in variant server	possible	*
PLEKHN1	1	Non-frameshift	PLEKHN1:NM_001160184:exon9:c.867_869del:p.289_290del, PLEKHN1:NM_032129:exon9:c.831_833del:p.277_278del,	-	possible	-
TTC3	21	Non-synonymous	TTC3:NM_003316:exon23:c.T2106A:p.D702E, TTC3:NM_001001894:exon23:c.T2106A:p.D702E,	Not in variant server	possible	*
ZNF829	19	Non-synonymous	ZNF829:NM_001037232:exon5:c.A294T:p.R98S	Not in variant server	possible	-
ESR2	14	frameshift deletion	ESR2:NM_001437:exon5:c.948delT:p.I316fs	Not in variant server	-	****
ZCWPW2	3	frameshift deletion	ZCWPW2:NM_001040432:exon6:c.684_688del:p.228_230del,	Not in variant server	-	**
SENP3	17	Non-frameshift deletion	SENP3:NM_015670:exon2:c.254_256del:p.85_86del	Not in variant server	-	**

Table 28. Targeted candidate analysis of alterations identified using an approach that excludes the de-novo paradigm. This analysis includes 17 non-frameshift alterations (non-synonymous and splicing) and 2 in-frame alterations. Both frameshift alterations that were common to the datasets were included here for further analysis.

Gene	Biological preservation	Known interactions	Candidate score
ESR2	Yes	Oestrogen receptor beta (ERbeta), isoform of oestrogen receptor (ER alpha). DNA binding domain within protein, multiple DNA binding sites via oestrogen response elements (ERE) including upstream of the RET gene. Ligand activated by oestrogen. Implicated in breast and thyroid cancers. Association with RET proto-oncogene in breast cancers refractory to oestrogen modulation (Stine et al., 2011).	*****
MED17	Yes	Loss of MED17 implicated in progression of prostate cancer due to abnormal androgen signalling pathways (Vijayvargia, May and Fondell, 2007). No known thyroid/neural crest interactions.	**
FOLR3	Yes	Folate receptors overexpressed in ovarian cancer (Yuan et al., 2009). Not implicated in thyroid disease.	**
TTC3	Yes	Ubiquitin protein ligase. Degredation of phosphor Akt. May play role in neuronal differentiation (Berto et al., 2014). Not implicated in thyroid disease.	**
NCKIPSD	Yes	Located at the nucleus, protein produce plays a role in signal transduction. Implication in leukaemia (Sano, 2001).	**
ZCWPW2	Yes	Zinc finger protein, implicated in cellular structural properties (Ilagan et al., 2014). Not implicated in thyroid of neural crest abnormalities.	***
SEN3	Yes	SUMO2/3 protease. Important role in post-translation protein modification. Implicated in many cancers including gastric and oral (Ren et al., 2014). No report thyroid/neural crest interactions.	***

Table 29. Targeted candidate analysis based on biological information excluding de-novo paradigm. The 7 alterations identified as most likely to be disease causing are further analyses here for potential biologically relevant interaction.

5.3.4 Identified mutations and rational for taking forwards

Based on the above analysis, mutations with the highest scores were further evaluated by capillary sequencing to ensure they were true positives. Confirmation of the ESR2 mutation (c.948delT) and ZCWPW2 (c.648_688del) along with the other deletions were confirmed by capillary sequencing (Figure 41/42). The SENP3 in frame deletion could not be confirmed by capillary sequencing and as such discarded from further analysis.

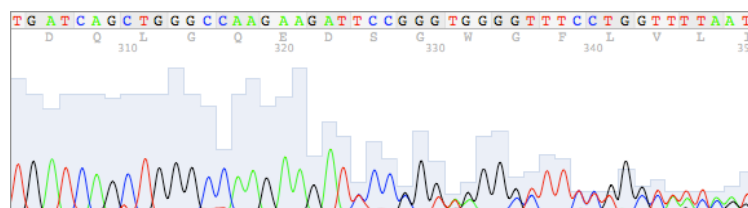


Figure 39. ESR2 c.948delT reverse

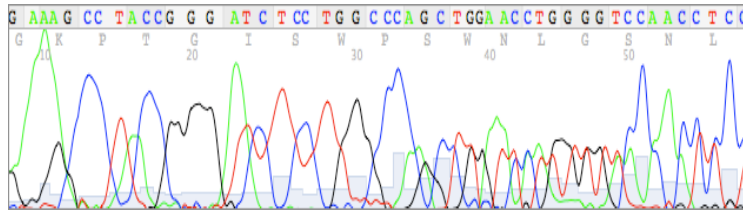


Figure 40. ESR2 c.948delT forwards

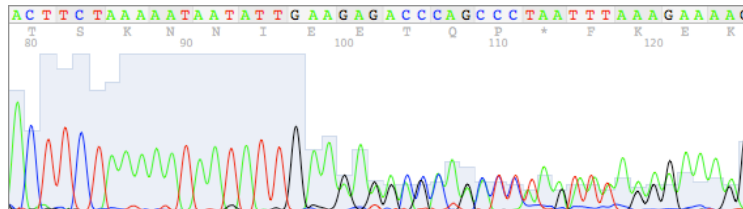


Figure 41. ZCWPW2 c.648_688del reverse

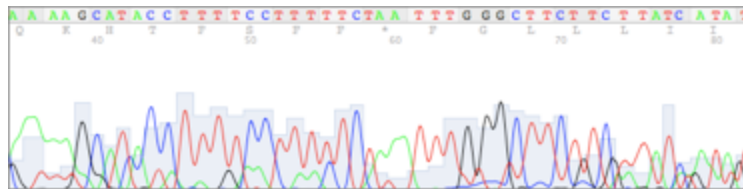


Figure 42. ZCWPW2 c.648_688del forwards

5.3.4 ESR2 and ZCWPW2: Biological assessment

The ESR2 gene encodes the trans-membrane nuclear receptor oestrogen receptor beta (ER β). ER β along with its isoform ER α are part of a superfamily of transmembrane and cytoplasmic receptors that regulate cellular transcription via complex intracellular signalling. ER β is expressed in thyroid tissue as well as breast and prostate. Oestrogen receptors (ER) bind 17 β -estradiol or related ligands which lead to the formation of ER homo- or heterodimers that interact with specific DNA sequences called oestrogen response elements (ERE) to activate transcription of specific genes. Recently, ERE were detected on the RET gene promoter and it was shown ER α was able to

regulate RET transcription (Zhao et al., 2007). ER β is thought to regulate the response of ER α and in this way may act directly as tumour suppressor gene, although the mutated form could also act as a proto-oncogene by binding directly to RET and leading to upregulation (Zhao et al., 2007). Further, the RET tyrosine kinase has been implicated in oestrogen independent breast cancer and those resistant to tamoxifen (Stine et al., 2011).

The ZCWPW2 gene encodes a zinc finger, CW type, with a PWWP domain. Zinc finger proteins bind DNA and RNA and act as stabilisers. They are extremely common in higher animals (found exclusively in vertebrates) and can be classified based on their structure and binding properties (Perry and Zhao, 2003). No phenotypic presentation of abnormalities in this gene has been described and its *in vivo* function is unknown. In terms of biological plausibility ESR2 gene alterations seem more plausible than ZCWPW2 and it is for this reason it will be taken forwards for further investigation.

5.4 Discussion

5.4.1 WES: Selecting mutations for in-vitro studies

WES has been successful within the context of this disease, generating a high level of fidelity (depth of base reading) and significant exome cover. By adopting a multi hypothesis approach to WES mutation analysis it has been possible to successfully reduce the number of candidate mutations to a workable group. Although this approach has been used before in the context of MTC, to date no causative mutations other than low frequency RET mutation have been discovered (Qi et al., 2011). The two mutations that scored highest in the bioinformatics analysis and were confirmed through capillary sequencing were ESR2 and ZCWPW2. As a biologically preserved, frameshift mutation in a relevant pathway, the c. 948delT ESR2 mutation is clearly the standout finding from WES. It was not possible however, to confirm the presence of SENP3 by capillary sequencing. Based on the stand out qualities of the ESR2 mutation we opted not to pursue the other non-synonymous mutations identified by the searches presented here.

Although an appropriate tool in the context of this study, WES is not without limitations even prior to the application of a bioinformatics approach to mutation filtration. As discussed, confining mutation searches to the exome alone may miss the mutation altogether (likely in 15% of cases) (Mamanova et al., 2010). The definition of the 'exome' has clear implications and although consensus on many gene loci have been reached, there are significant differences between sequencing platforms (Kim D Pruitt et al., 2009).

Furthermore, within platform considerations such as target regions not well covered by the sequencing technology, clearly has the potential to falsely eliminate target mutations prior to application of bioinformatics approaches (Biesecker, Shianna and Mullikin, 2011b). These points considered, WES is the most applicable methodology to use within this study.

An incomplete understanding of the biology of the disease under investigation represents a potential problem. An attempt has been made here to perform multiple search strategies of the same dataset under differing criteria to reach a 'consensus' search that is inclusive of the mutations that require further interrogation without including so many as to be impractically workable. Other authors use similar approaches, applying phenotypic and heredity characteristics into a bioinformatics based search approach (Comino-Méndez et al., 2011), (Robinson, Krawitz and Mundlos, 2011). The first consideration is that of commonality. The assumption that all members of a cohort that phenotypically present with disease carry the mutation and that those unaffected do not, was made initially. Ideally, in familial studies, the most distantly related members of the same family would be chosen for WES to increase the likelihood of mutations common to both or multiple individuals are more likely to be disease causing (signal) and reduce the number of non-relevant mutations (noise) (Figure 37) (Michael J. Bamshad et al., 2011). This is less easy to achieve in small families such as the one presented here. The risk with commonality assumptions is that mutations may not be fully penetrant or, at least when sampled, individuals did not have disease specific signs or symptoms (Summerer, 2009). In these data presented here, the

novel hypothesis was rejected, indicating that one of the proband's parents was a carrier of all mutations deemed significant.

In relation to RET mutations, it is estimated that penetrance is in the region of 80% but worth bearing in mind the carrier status of RET sequence alterations in the general population is as high as 10% (Frank-Raue et al., 2011). Further, commonality (individuals with the same phenotype sharing the same alteration) is an important factor when considering multiple individuals from differing pedigrees, although less of a problem when sampling multiple individuals from the same pedigree, as modifying factors from the expression of different genes would likely be less prevalent (Figure 36).

The second factor to consider is presumed mode of mutation transmission. Typically, diseases of autosomal dominant pattern of inheritance present with multiple affected individual within a family by vertical presentation, such as the one observed in our index family (Newman et al., 1988). Autosomal recessive disorders on the other hand present with a pedigree where phenotype is seen in a horizontal pattern (Eng et al., 1996). Furthermore, if the trait is dominant then one would expect approximately 50% of an affected individual's offspring to be affected rather than 1 in 4 for recessive traits. In rare disease, autosomal dominant patterns of inheritance are more common from a heterozygous mutation. Assuming a dominant pattern of inheritance allows removal of homozygous mutations from datasets and therefore further analysis (Michael J. Bamshad et al., 2011).

The third assumption is that a mutation is derived do-novo. It is understood that a large proportion of the heritable disease encountered clinically cannot be explained by data accrued from population based genomic studies such as genome wide association studies (GWAS) (Stringer et al., 2011). It is therefore postulated the shortfall between what is predicted and observed is made up from de novo mutations acquired at meiosis through replication fidelity errors in potentially tumorigenic genes, each individually too rare to be detected by GWAS but having a large effect on disease prevalence (Ryoo and Lee, 2014) (Manolio et al., 2009). Further, mutation 'novelty' should be assumed in bioinformatics approaches in gene mutation discovery as non-novel mutations, that is, those previously described in other forms of disease and presented on the various mutation servers, would presumably have already been linked to MTC if mutations were found to be causative (1000 Genomes Project Consortium et al., 2010). Removing all SNPs that are previously described in SNP databases further reduces the number of mutations that can be excluded.

Further assumptions were made based on the likely effects that a mutation would have on the transcription process to mRNA and ultimately the protein product. Synonymous point mutations (leading to a change in the coding DNA a single point but, although a different codon is transcribed, the same amino acid is produced) were excluded. Non-synonymous mutations were included although the predicted effect of this type of single base mutations is extremely variable. Modelling through online tools (SIFT and Polyphen) was performed in these situations to predict potentially damaging effects (Adzhubei, Jordan

and Sunyaev, 2013). The mutations most likely to alter protein structure are those where there is either a significant change in the code due to deletions or insertions or where a stop codon is either lost or gained (Lengauer, Kinzler and Vogelstein, 1998). Deletions and insertions can be well tolerated if they lead to in-frame change and loss or gain of a single amino acid coding region. Where insertions or deletions are out of frame however, this can lead to truncation or elongation of a transcriptional region and therefore protein (Vogelstein and Kinzler, 2004). It is to these mutations special attention was given when identifying potential candidates.

5.4.2 Exome data and mutational analysis

ESR 2, encodes oestrogen receptor beta (ER β), consisting of a 9 coding exon gene with multiple isoforms found on chromosome 14. It was discovered in 1996 using degraded primers in studies of ER α with which it shares significant homology, particularly in its DNA binding domain. The most common two identified isoforms of ESR2; isoform a (wildtype, 530aa) and isoform b (496 aa), differ in exon 9 coding (ligand binding domain). The c.948delT frameshift deletion leads to a coding of a further 26aa chain before a stop codon is reached (aa position 330). The mutation is predicted to affect the AF2, ligand binding domain indicating the possibility of a protein that degrades due to nonsense mediated decay or one that functions without the ligand binding capabilities.

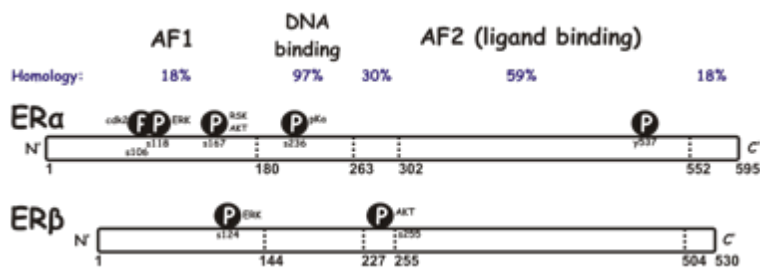


Figure 43. ER homology

ERα and ERβ form homo and heterodimers on ligand binding with oestrogen (E2) and its analogues. Once dimerised by ligand, ERs act via classical pathways, binding to specific DNA sequences known as oestrogen response elements (EREs), to activate transcription specific genes (Ogawa et al., 1998). By altering chromatin structure and up-regulating gene expression, ER bound EREs ultimately lead to cellular growth and differentiation. EREs have been identified in the upstream regions of many genes, and recently, EREs have been detected on the RET gene promoter. It has been shown that ligand bound ERs[7] regulate RET transcription and thus cellular proliferation (Zhao et al., 2007).

ERα, encoded by the ESR1 gene, was the first ER to be discovered (1986) and studied in great detail in the context of breast cancer where it forms part of the prognostic work up of breast tumours and a marker, as well as direct target, for oestrogen modulation therapy most commonly in the form of selective oestrogen receptor modulators (SERMS e.g. Tamoxifen) (Clemons, Danson and Howell, 2002). ERs contain the structural, functional domains A-F. The A/B-domain contains the activation function 1 (AF-1), which is

hormone/ligand-independent. The C-domain contains the DNA-binding domain, with the hinge region in the D-domain having nuclear localization sequences that interact with AP-1. The E/F-domain is the ligand-binding domain with ligand-dependent activation function, AF-2 (Burns and Korach, 2010).

ER β , has been proposed as a tumour suppressor gene and *in vivo* is thought to exhibit a dominant negative effect, inhibiting the transcriptional up-regulation of ER α , thus inhibiting the physiological response of oestrogen. Further, ER β has been shown to repress c-myc, cyclin D1 and cyclin A and to increase the expression of p21waf1/cip1 and p27kip1 causing a cell cycle arrest of MCF-7 cells in G2 phase (Paruthiyil et al., 2004). ER β is expressed ubiquitously throughout the body including thyroid tissue as well as breast, prostate, colon and brain (Taylor and Al-Azzawi, 2000). Further, ER β has been shown to be expressed in both thyroid follicular and parafollicular C-Cells whereas ER α is not expressed at all (Bléchet et al., 2007). Conversely, in MTC, ER α is reported in as many as 90% of tumours with expression of ER β more variable, typically around 70% but often much lower (Taylor and Al-Azzawi, 2000). I propose that the down regulation of ER β and possible up-regulation and unopposed action of ER α are important events in C-cell proliferation and could potentially lead to malignant predisposition via the intermediary CCH. It follows that such a change could be oestrogen mediated and involve up-regulation the RET pathway.

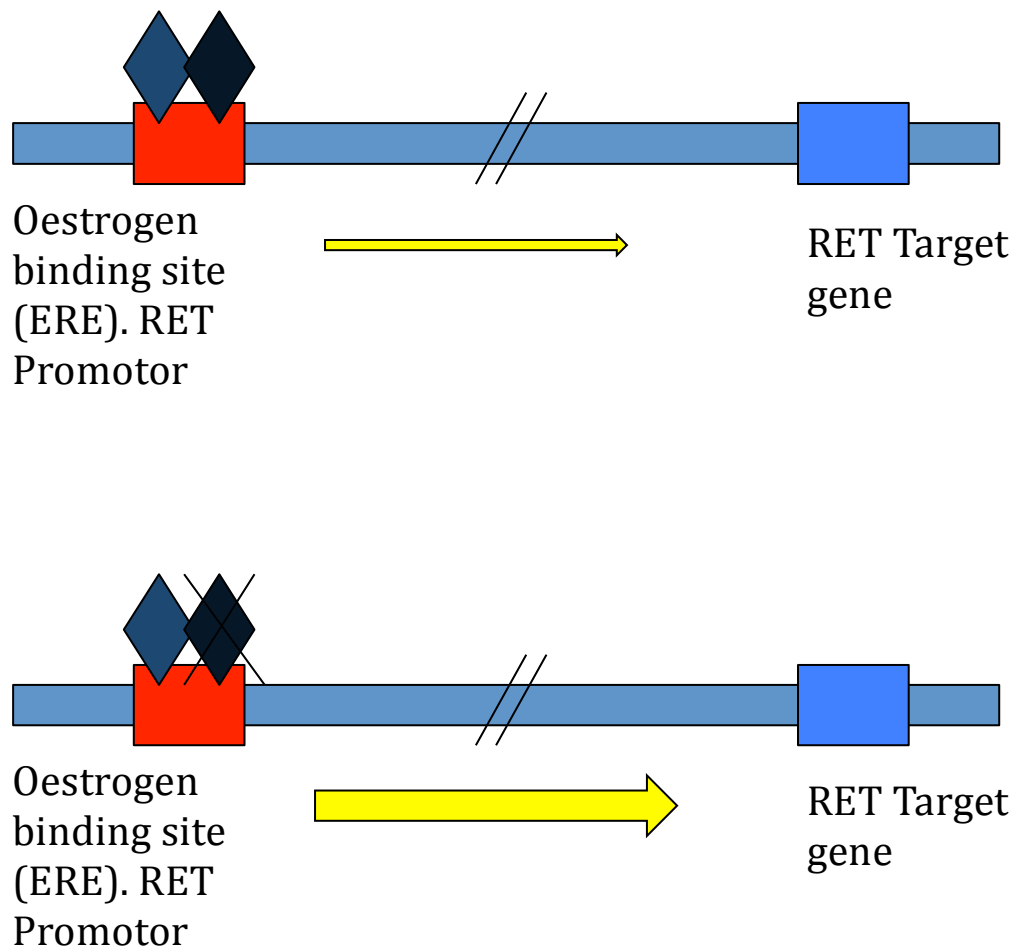


Figure 44. ESR 1 and 2 protein products ($ER\alpha$ and $ER\beta$) on ligand activation bind to oestrogen response elements (EREs) one of which has recently been discovered in the RET promoter region. It is postulated that the c.948delT mutation leads to a null or attenuated protein product, reducing the inhibition of $ER\alpha$ induced RET up-regulation.

The potential link between oestrogen signalling via ERs in thyroid disease and thyroid cancer has recently been suggested (Rajoria et al., 2012). The concept is however not novel, with studies as early as 1988 demonstrating *in vitro* TT cells (MTC cell line) proliferation in response to oestrogen and growth inhibition in response to tamoxifen (Yang, Pearson and Samaan, 1988). Oestrogen has been shown to mediate cellular proliferation via ERs in other thyroid cancer cell lines with ligand bound $ER\beta$ responses via EREs typically leading to decreased proliferation on MTT assays (Zeng et al., 2007). Furthermore, an anti-oestrogen therapy has been proposed as a treatment for

thyroid malignancies, although to date no clinical trials have been undertaken (Rajoria et al., 2012). Based on sequencing results and these biological interactions, we believe the c.948delT mutation of ESR2 to be the most appropriate candidate gene to take forwards for further investigative functional work.

5.4.3 Proposed experiments based on WES results

Aims

- To assess the wider cohort of familial and sporadic disease for the presence of ESR2 mutations
- To model ESR2 mutation *in vitro* in appropriate cell lines
- To demonstrate the ESR2 c.948delT mutants lead to null proteins
- To demonstrate the expression of mutant ER β leads to up regulation of the RET pathway.
- To demonstrate the effect of specific oestrogen receptor antagonists on both proliferation and on RET pathway up regulation
- To establish ER β and ER α immunostaining patterns in familial and sporadic disease

To assess which cell lines are most appropriate for use within these experiments, the following cell lines were cultured to extract protein and test ER α and ER β positivity by western blotting.

MCF 7: Breast cancer derived cells expressing both ER α and ER β

T47D: Breast cancer derived cells that express neither ER α nor ER β

TT cells: MTC derived cells, ER positivity to be determined

Although MCF7 and T47D are breast rather than thyroid cell lines, we believe they may be good to use as preliminary models both due to their expression characteristics with regard to ERs and also as they are easily transfected and grow rapidly. TT cells are the only MTC model and are derived from the metastasis of a patient with disseminated disease and a known RET mutation (C634W: MEN2A). This may confound our assays of RET and RET pathway in this line although as the only *in vitro* model of MTC we feel its use is justified.

Chapter 6.

ESR2 sequencing of familial and sporadic MTC

6.1 Introduction

Having identified a candidate mutation from WES, and confirmed it through capillary sequencing, the next step was to confirm the presence of c. 948delT ESR2 mutations in the family and wider cohort of sporadic patients recruited with MTC. The primary question is whether the mutation segregates within the family (and also to possibly assess penetrance). The second question was to examine whether further mutations in germline and tumours were present in the remaining ESR2 exons. Clearly, when dealing with a possible disease susceptibility mutation, a complete lack of segregation within the family would indicate reconsideration of the identified candidate genes or the consideration of complex penetrance issues.

As discussed, one hypothesis regarding the biological behaviour of ER β is that it acts as a tumour suppressor. As a heterozygous mutation (c. 948delT ESR2), and if adopting a classical theory of tumour suppressor genes, one would expect a second hit in the same gene to confirm cancer predisposition (Knudson, 2002). The second hit could be present in either germline or, more likely, in somatic DNA and as such both should be examined for the presence of mutations throughout the ESR2 gene. If no second hit was identified, it is possible that the c. 948delT ESR2 mutation is still the driver mutation for MTC predisposition in the family but that a haploinsufficient or 'mutator' phenotype model is more likely (Loeb, 1991). It is possible however, that no other mutations will be identified and again, in this case we would have to consider returning to our list of candidate genes to explore other possibilities.

Understanding the prevalence of ESR2 mutations in the wider cohort of both germline and somatic DNA would insight into the comparative mutation profiles and there relative frequencies. Finding additional mutations in the wider cohort would also add weight to our theories regarding ESR2 c. 948delT driven MTC predisposition and the relevance of ESR2 mutations in sporadic disease. Again, it has to be accepted at this stage that, as a potential causative mutation in a rare family phenotype, there may be very few germline or sporadic mutations of the ESR2 gene. If this is the case then decisions regarding the on-going functional work will need to be made.

6.2 Materials and methods

6.2.1 Histology and tumour blocks

Histological specimens were obtained from all members of the family who had undergone thyroid surgery, where available. Newly cut and slides were cut from tumour blocks as needed. Histological classification and examination of slides were performed independently and with the examiner (RB) blinded to the clinical information. All histological classifications were cross-referenced with original histological diagnosis and where there was disparity, secondary checks and a second reviewer examined the slides. In two cases there was a change in the eventual pathological diagnosis. In the original examination of the prophylactic thyroidectomy specimens from two of the offspring of the proband, one had been classified as CCH but was reclassified normal and one normal case classified was reclassified CCH. In the original examination of specimens, calcitonin staining had not been performed, which accounted for the discrepancy.

6.2.2 PCR and sequencing

PCR amplification of individual exonic regions of the ESR2 gene in germline and somatic DNA samples was performed for all patients using methods as previously described for RET exonic sequencing. Again, primer pairs were designed for each exonic region using Primer 3 software as previously described. Where PCR bands were not clearly identified using these methods primers were redesigned.

Oligonucleotide	Sequence	Redesign needed
ESR2 Exon 1 Forwards	GCGCTTACCTCGCTTCAGT	-
ESR2 Exon 1 Reverse	ACAGAAAGGCGCTTCTGAAC	-
ESR2 Exon 2 Forwards	TTCTCACCATCCCTCACTCA	TTTTTGCCTTGAAGAAGCCTTA
ESR2 Exon 2 Reverse	GTGTCAGCGGCTGTGATAAG	-
ESR2 Exon 3 Forwards	CCCCACAGACCTGACTTCTC	-
ESR2 Exon 3 Reverse	GAGCAAGACCAGCAGTAGCA	-
ESR2 Exon 4 Forwards	CAAAGTGCAGGCTGTGCTC	CAAAGTGCAGGCTGTGCTC
ESR2 Exon 4 Reverse	ACACTGGGTAGACCACAGCA	GACCGAGAAACGAACTGTGG
ESR2 Exon 5 Forwards	ACTGACCAACGCCCTCTG	GGACGTGCAGCATTCTAAGG
ESR2 Exon 5 Reverse	AAGAGCGAGCACCTCATTTT	-
ESR2 Exon 6 Forwards	AGAGCAGCTTGGTGGTCATT	ACATGAGGAAGCAGCCAGAG
ESR2 Exon 6 Reverse	CCCCAGACAGGCAATAGGTA	GTCACCTGCCTCCCTGTG
ESR2 Exon 7 Forwards	AGCTGCCTGGCTAAGGTGT	TACCCTCAGGCCATTACAGG
ESR2 Exon 7 Reverse	AGGCCAGGCTCCAGAAG	GCTTTTCTCAAAGGGCAGGA
ESR2 Exon 8 Forwards	CCTGTGACCCTGCTTGCT	-
ESR2 Exon 8 Reverse	AGACCTGGAGCAGGGAGAG	-
ESR2 Exon 9 Forwards	GATCTGCCTAGGAGGTGGTG	GATCTGCCTAGGAGGTGGTG
ESR2 Exon 9 Reverse	GTTCCCATGCCCTGATTAAA	TCTCCTACCTGTTCCCATG

Table 30. Oligonucleotide primer designed for the sequencing of ESR2

6.3 Results

6.3.1 Germline mutations

6.3.1.1 The ESR2 c. 948delT mutation segregates in the family to individuals with disease proven by histology

All cases of histologically confirmed CCH and MTC, after review were found to have germline mutations in the ESR2 gene corresponding to the c. 948delT ESR2 mutation. In addition, the father of the proband also had the c. 948delT ESR2 mutation but has not developed MTC (Figure 46). Calcitonin testing showed elevated results but based on this alone and in the absence of RET mutation, no further investigation or treatment was carried out.

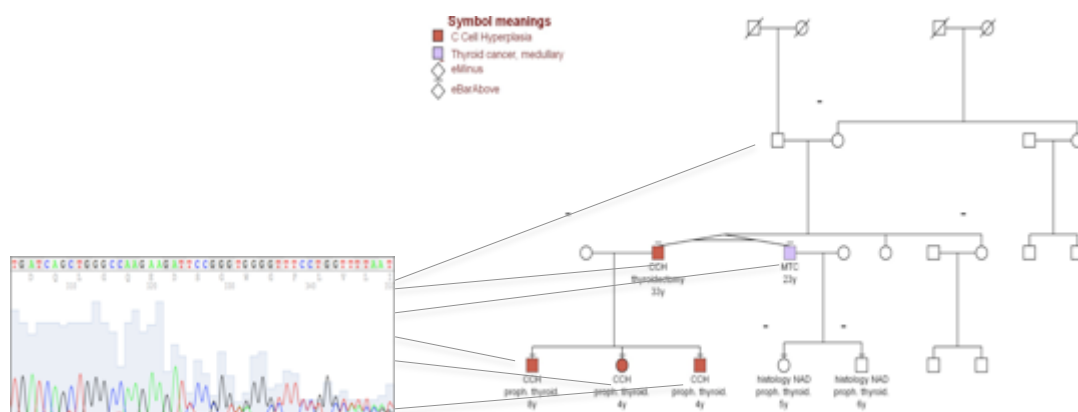


Figure 45. Sequencing confirmation of individuals carrying the germline c. 948delT mutation ESR2 confirmed by sequencing

6.3.1.2 No germline ESR2 mutations were found in other recruited families

ESR2 sequencing was carried out on 12 individuals across the two additional families shown in Figure 22 (page 91). Neither the c. 948delT ESR2 mutation

nor any other mutation in the ESR2 gene has been identified in either of the two families for which we had germline DNA available at the time of the study (Figure 22, page 90, families recruited to the study in collaboration with MR, Madrid). The more recently recruited families are awaiting testing but these results will be reported elsewhere.

6.3.1.3 Germline ESR2 mutations in the wider cohort

A single novel germline alteration (ESR2 c.800 C>G, p. V128L) has been identified in a patient with young onset sporadic disease. At the time of writing, this alteration has not been reported in SNP databases. Whether this is a novel mutation or rare SNP is not clear but given its context within patients with young onset sporadic disease, warrants further examination. Modelling this alteration in SIFT and Polyphen suggests that this alteration is well tolerated.

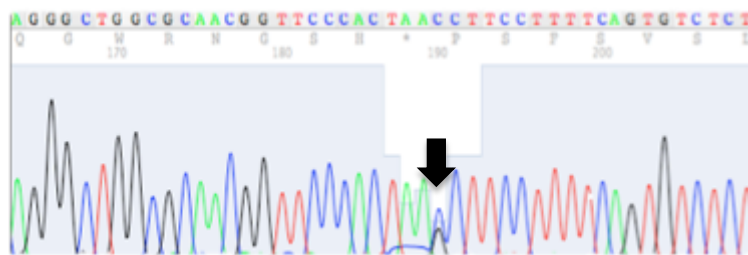


Figure 46. ESR2 Exon 3 forward c. 800 C>G p. V128L

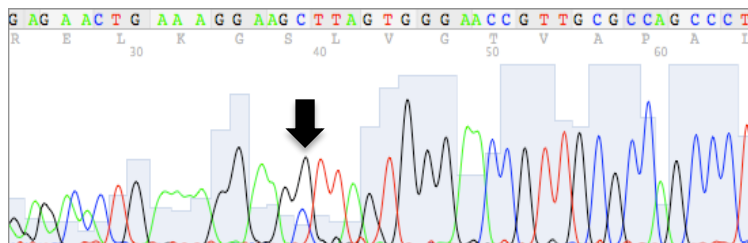


Figure 47. ESR2 Exon 3 reverse c. 800 C>G p. V128L

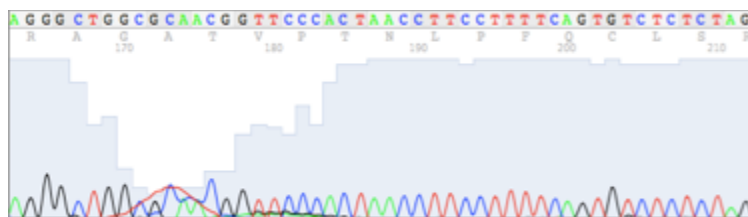


Figure 48. ESR 2 Exon 3 Normal sequence

6.3.2 Somatic mutations

Somatic alterations of the ESR2 gene were identified in DNA extracted from two tumours from patients from Sydney within the splice site of Exon 9 c. 917 T>C p. L306S (Figures 50-52).

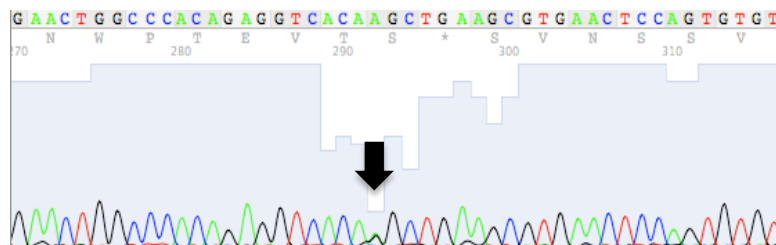


Figure 49. Splice site exon 9 forwards c. 917 T>C p. L306S

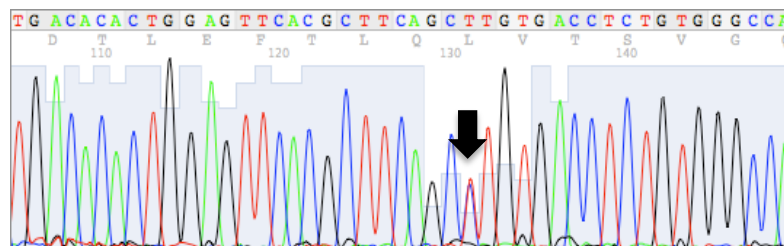


Figure 50. Splice site exon 9 reverse c. 917 T>C p. L306S



Figure 51. Exon 9 normal

6.3.2.2 No further germline ESR2 mutations been discovered to date

In all, 53 germline samples of DNA and 24 samples of somatically derived DNA were analysed for ESR2 alterations. Of the germline samples, 36 had apparently sporadic MTC, with 12 familial MTC positive disease (individuals with histologically confirmed CCH/MTC from three families) and 5 familial samples with no clinical evidence of disease (unaffected members of the

index family). In all, novel alterations were identified in 7/54 germline DNA samples (c.948delT in six members of the index family and p.V128L in one young onset sporadic MTC) and 2/24 tumour samples (p. L306S in two Tumours from Sydney).

Patient type		N=	Germline alteration	ESR2	Somatic alteration
Familial RET CCH/MTC	Non-	12	5/12		-
Familial RET (No clinical phenotype)	non-	5	1/5		-
Apparently sporadic RET MTC	Non-	36	1/36		0/12
Somatically derived DNA		24	-		2/24

Table 31. Identified ESR2 alterations in familial and sporadic MTC recruited to the study.

6.4 Discussion

The fact that the disease phenotype and c. 948delT mutation segregate within the family and correlate with histology is a positive finding adding weight to the hypothesis that ESR2 mutations predispose to CCH/MTC. The presence of a mutation within, as far as can be ascertained, an unaffected family member is potentially problematic. Several explanations may exist. Firstly the mutation is incompletely penetrant and at that generational level there exist modifying genetic influences that abrogate the effects of the mutation, possibly by epigenetic events (intergenerational gene silencing) (Harper, 2005), (Orozco et al., 2014). RET mutations themselves are estimated to be around 80% penetrant and as a novel mutation it is impossible to predict what the penetrance might be (Frank-Raue et al., 2011). It is unlikely to be 100%. Secondly, we assume that CCH is always a premalignant condition and that ultimately the children who underwent thyroidectomy prophylactically and were found to have CCH would have gone on at some point to develop MTC. This is not necessarily the case and we do not know to what extent if any environmental factors have played a role in the development of MTC in the proband e.g. second hit or other epigenetic event. It is worth noting that the proband's brother (identical twin) had CCH only with no evidence of MTC on histology. Thirdly, the father of the proband had had an elevated calcitonin following pentagastrin stimulation so it is entirely possible that he too has CCH on histological grounds although we will not know for sure. Based on these findings within the family, we feel it is reasonable to take forward ESR2 c. 948delT for *in vitro* interrogation.

Finding a novel alteration (c.800 C>G p. V128L) in the germline of a patient with young onset disease is also potentially interesting and adds weight to the theory that ESR2 mutations are important in MTC tumourigenesis. Taken in isolation this could be a rare SNP and have no relevance to MTC predisposition. Indeed the modelling by SIFT and Polyphen-2 data-bases would predict that Valine to Leucine residue changes are not likely to be detrimental (Adzhubei, Jordan and Sunyaev, 2013). However, the patient is young and the mutation is in the germline rather than tumour and it is in a highly conserved region of the exome. As seen from the wider cohort, several SNPs are highly prevalent, but this one is neither prevalent in our sample nor when searching the wider database. Furthermore, modelling databases such as Polyphen 2 are modelled on sequence and protein property algorithms and have an inherent false negative rate (Adzhubei, Jordan and Sunyaev, 2013). Single residues suspected of being important cannot be truly predicted using bioinformatics databases but rather need to be scrutinised in the laboratory. Within the context of this study, and with the clinical features, it was deemed sensible to model this mutation and take it forward for further investigation.

It is harder to establish the possible effects of the splice site mutation in the somatic DNA of the patients from Sydney. Again, it is a novel mutation but cannot easily be modelled *in vitro*. To this end I have excluded it from further functional work but may revisit at a later date depending on the functional results.

In total, I have identified three novel mutation in the ESR2 gene in RET negative patients with MTC. In the absence of other strong candidates I feel that this is sufficient evidence to explore ESR2 functionally to attempt to unpick the biochemical pathways. The fact I have not identified further mutations may be representative of inappropriate candidate gene selection. A further possibility is that, as discussed earlier in this report, candidate genes other than RET are likely to exist in low frequencies. Access to a wider cohort, particularly of sporadic patients with young onset disease is likely to have significant impact. I am however obliged to progress with the project in the most appropriate way and accept that some of the assumptions I have made can only be tested by *in vitro* functional evaluation.

Chapter 7.

Functional studies

7.1 Introduction

The functional work presented here has two main aims. First is to link, through cellular modelling, the interactions between ESR2 mutants and proliferation pathways including RET at the level of mRNA and protein expression. Second is to link ESR2 mutations to the index family and sporadic cohort. Having agreed to move forwards with ESR2 as a candidate predisposition gene, plasmid vectors were created containing ESR1 (Wild type oestrogen receptor alpha: WT ER α), ESR2 (in two common isoforms WT ER β a and WT ER β b), and ESR2 c. 948delT and ESR2c.800 C>G p.V128L mutations for *in vitro* experiments (see Chapter 2). Finally, in the study of hormone receptors it is clearly important to unpick some of the oestrogenic effects that may influence target gene expression, as the ligand driver of receptor binding and downstream up-regulation, is an important consideration and has been included in the analysis here.

The primary hypotheses are that:

- Identified mutations of the ESR2 gene lead to null or attenuated ER β proteins
- That ER β mutants lead to cellular dysregulation and predispose to proliferation compared with wild type WT_[8] and thus potentially to malignant change
- As a tumour suppressor gene, ESR 2 null mutants lead to loss of inhibition of ESR 1 driven up-regulation
- That a haplo-insufficient model of tumour progression is present in this family leading to cancer predisposition

- That cellular deregulation is driven by alteration in the RET pathway.

7.1.1 Cell lines

The choice of a suitable model is clearly important. There are a number of key considerations when considering MTC and the options that are available. Human derived MTC cell lines are available, the most readily used being the Human TT cell line although others such as the MTC-SK cell line have been developed, they are less widely used (LCG Standards, USA (Anon, n.d.)). The TT cell lines were established from a needle biopsy from a 77 year old Caucasian female with aggressive, but presumably sporadic (based on age at presentation) form of the disease (Berger et al., 1984). The cells line was subsequently found to harbour the codon 643 cysteine to tryptophan (C>T) missense mutation most commonly associated with the MEN 2a (Carlomagno et al., 1995). This has significant implications for this study as we are essentially examining biochemical pathways important in cancer predisposition in patients without RET mutations. As one of our key outcomes in trying to link ER β expression to RET tyrosine kinase expression, choosing a model with endogenous, abnormal RET expression is problematic. Furthermore, many authors report significant challenges in transient transfection of TT cell in culture due to their relatively slow growth (doubling time 83 hours) and difficulty establishing, non-adenoviral vector transfection (Behr et al., 1997). However, as most studies in MTC have utilised this cell line we believe that an attempt to include TT cells in a battery of cell lines would be prudent, even if we would not pursue this if difficulties were encountered.

Although primary culture of malignant cells derived from MTC has been reported, primary culture of C cells has not and would have a number of technical issues. The position of C cells within the thyroid gland and their paucity in normal tissue, render cellular isolation difficult and the likelihood of contamination with other cellular components of the thyroid gland including thyrocytes and lymphatic components is high (ROOS et al., 1975). Primary cell culture of C cells was considered in the context of this study but was beyond what was achievable in the allotted time frame. Primary culture of C cells would be the ideal model for the experiments described here, however, and as such should be considered in the further planned work section of this report.

With these issues in mind, in addition to TT cells, the following cell lines were chosen for culture preparation and attempted *in vitro* experimentation.

7.1.2 MCF 7

MCF 7 are adenocarcinoma of the breast derived from cellular material taken from metastatic pleural effusion of a 69 year old Caucasian female (Soule et al., 1973). MCF 7 cells are widely used in breast cancer as well as generic cancer biology and widely used in the study of oestrogen and androgen signalling responses and oestrogen receptor studies. MCF 7 cells, in addition to normal breast tissue and other breast tumours, express both ER α and ER β in varying amounts (Vladusic et al., 2000). MCF 7 cells readily transfect and rapidly divide, making them an ideal starting point for protein and mRNA work

and for our planned work with oestrogen signalling and pathway upregulation. The interaction between these two receptor isoforms in MCF 7 cells is thought to be important in cellular proliferation, with ER β acting to inhibit growth in a ligand dependent and independent manner (Treeck et al., 2010) (Paruthiyil et al., 2004). Endogenous expression of ER α and ER β is a limitation of this cell line, and up regulation in these already activated pathways by transient and stable transfection, will have to be measured over and above endogenous expression.

7.1.3 T47D

The T47D cell line is another breast cell line and like the MCF 7 cell line, is derived from a malignant pleural effusion and variably expresses ER α and ER β (Lu et al., 2005). T47D cells are again readily transfectable and have a rapid doubling time, making them a suitable comparator for the MCF7 cell line. An advantage of the T47D cell line over that of MCF 7 is the absence of the RET/PTC rearrangement, with the primary expression characteristics being the WNT7B oncogene.

7.1.4 mRNA expression

The measurement of target gene expression by measuring mRNA is an established technique in experimental and diagnostic fields and has the ability to provide quantitative data at the level of gene expression from a very small number of cells. Ultimately, measuring mRNA expression is a tool to measure the decision-making within cells under certain conditions and allow

assessment of a multitude of parameters regarding cellular differentiation and proliferation at the same time (Zamorano, Mahesh and Brann, 1996). Within the context of this study, I am more interested in the level of functional protein produced in the context of transient manipulation of gene expression. However, mRNA expression adds valuable information to support these hypotheses as well as allowing a more detailed investigation of the more subtle interaction between the genes of interest and down-stream pathways. Clearly, taken on its own, the quantification of mRNA expression following transient transfection does not establish a direct link between genes of interest and cellular behaviours as multiple steps following gene transcription will dictate whether functional proteins will be produced. mRNA expression data does have the potential, however, to add weight to some of the theories regarding the interactions between genes and to the extent that these interactions are ligand driven (Bustin, 2000).

Reverse transcription polymerase chain reaction (RT-PCR) can involve either a one or two step technique and when combined with Taqman chemistry, can give reliable real time information regarding mRNA expression. The fundamentals of the procedure involve the creation of a cDNA library from RNA extracted under various experimental conditions prior to conventional PCR in combination with a labelled probe. In this case, specifically designed Taqman probes measuring (Applied Biosystems, Paisley, UK^[9]). Reverse transcription was catalysed using avian myeloblastic virus reverse transcriptase (AVM-RT: Applied Biosystems; Paisley, UK) which has higher fidelity, reducing issues with RNA secondary structure than other reverse

transcriptases (Freeman, Vrana and Vrana, 1996). As cDNA libraries are much more stable they can be stored at -20 °C and revisited as needed.

The Taqman assays for ER α , ER β and RET were obtained and used as per manufacturers guidelines (Invitrogen; Paisley, UK). The assays consist of a fluorescence labelled dye and a quencher on either end of probes designed specifically to the '5 end of the amplicon (Holland et al., 1991). Primers are designed for either end of the amplicon on separate exons. During thermal cycling, taq polymerase reconstitutes the amplicon. As the fluorescent probes have a lower affinity than polymerisation, the probe is displaced and hydrolysed, first at the fluorescence end and then at the quencher end of the probe (Bustin, 2000). As the dye and quencher are separated the fluorescence intensity can be measured and is directly proportional to the number of molecules that bound probes during the cycle (Bustin, 2000). Fluorescence intensity machinery is specifically designed to perform thermal cycling as well as fluorescence detection and quantification. As the PCR amplification is very sensitive at amplifying small quantities, contamination remains a key issue in setting up experiments. The single analyser and cycler set up, allowing reactions to take place in a single sealed well of a 96 well plate without the need to potentially introduce contamination goes some way to ameliorating this. (Bustin, 2000).

7.1.5 Immunohistochemistry

It is important within the context of a clinical based study such as this to make an attempt to tie back the findings in the laboratory with the clinical

parameters and observation with blood and tissue samples collected so far. Immunohistochemistry is a technique that, used in isolation, can have considerable issues with regard to establishing scientific basis of diseases (Tavangar et al., 2007). It is, however, a useful technique in some situations and can provide valuable corroborative evidence regarding causative links. The major limitation to the use of immunohistochemistry within the context of this study is the reliability (sensitivity and specificity) of the staining techniques in verifying true positives and negatives particularly in the context of historical blocks and slides. Particular issues arise when using staining techniques and looking for protein expression beyond that which is used in the diagnostic setting and in addition, using antibodies that are not optimised for a specific tissue .

With respect to the tumour library available for this project, the most interesting results would be to look at the ER β characteristics between family members with a known ER β mutation and the wider cohort of patients with MTC, as well as looking specifically at RET expression levels in relation to this. Neither RET nor ER β staining is optimised for use within the clinical setting and as such, challenges exist in extraction of meaningful and comparable results.

7.2 Methods

7.2.1 Cell culture

Cells were maintained and passaged as described in Chapter 2.

7.2.2. Site directed mutagenesis

Primers were designed as previously described to induce the two ESR2 mutations identified within the cohort. PrimerX software was used to model the identified mutations (web based SDM primer designing tool; bioinformatics.org, USA) and optimised using for melting temperature and GC content to improve binding capability using the following equations.

$$T_m = 81.5 + 0.41(\%GC) - (675/N)$$

$$T_m = 81.5 + 0.41(\%GC) - (675/N) - \%mismatch$$

Primers were purified using polyacrylamide electrophoresis (PAGE) to improve fidelity prior to use.

Primer Name	Sequence	Melting Temp °C	% GC content	Flanking region	Mutation	Purification type
948delT forward	GGCCAAGAAGAT CCCGGCTTTGTG G	77.8	60.0	12bp	Single base deletion	PAGE
948delT reverse	CCACAAAGCCGG GATCTTCTTGGC C	77.8	60.0	12bp	Single base deletion	PAGE
V128L forward	GACACTGAAAAG GAAGCTTAGTGG GAACCGTTG	77.7	48.5	16bp	Single base substitution	PAGE
V128L reverse	CAACGGTTCCCA CTAAGCTTCCTTT TCAGTGTC	77.7	48.5	16bp	Single base substitution	PAGE

Table 32. Site directed mutagenesis primer design

7.3 Results

7.3.1 MCF 7 cells were the most reliable cell type for ER receptor experiments

After multiple attempts and obtaining cells from multiple sources we were unable to establish TT cells (MTC cells harbouring RET c. C643T), in a stable condition to undergo transfection. Owing to time, the TT cell line was eventually abandoned. Significant Issues with T47D and Hela cell models were encountered in establishing stable expression of wild type ER β following transfection (data not shown). Although endogenous ER α /ER β were not detected in any of the cell lines, WT transfection in MCF7 showed consistently detectable up-regulation following transfection and as such form the backbone for the protein and mRNA work shown here.

7.3.2 Myc Expression of c. 948delT ESR2 mutation confirms a null protein using N terminal tagged plasmid in MCF 7 cells

Initially, C terminal tagged plasmids were used to transiently transfect MCF7 cell lines in stable culture. As the c.948delT mutation would be predicted to produce a truncated protein, non-detection of the C terminal tag would be expected and as such further vectors were established with N terminal tags. Figure 53. shows transfection with vector only control, ER α WT, ER β WT, p. V128L and c. 948delT over time. Maximum protein expression was seen at 24 hours. No expression of c. 948delT is seen at any point following transfection with C terminal and N terminal tagged plasmids. Expression of the p. V128L

mutation appears attenuated compared with wild type. Beta actin is shown separately and displays no disparity in terms of protein quantities. Data shown here was repeated in triplicate with both types of plasmid backbone.

Under the same transfection conditions and again using myc N-terminal tagged plasmids, immunofluorescence demonstrates nuclear localisation of WT ER β (Figure 54). Significant proportions of cells expressed ER β following transfection indicating a high rate of transfection success when cross referenced with non-specific nuclear staining (4',6-diamidino-2-phenylindole DAPI: Life Technologies, Paisley, UK). A notable decrease in response was noted with ESR2 p. V128L mutations, with no MYC expression was seen in MCF 7 cells Transfected with ESR2 c. 948delT mutations (Figure 54).

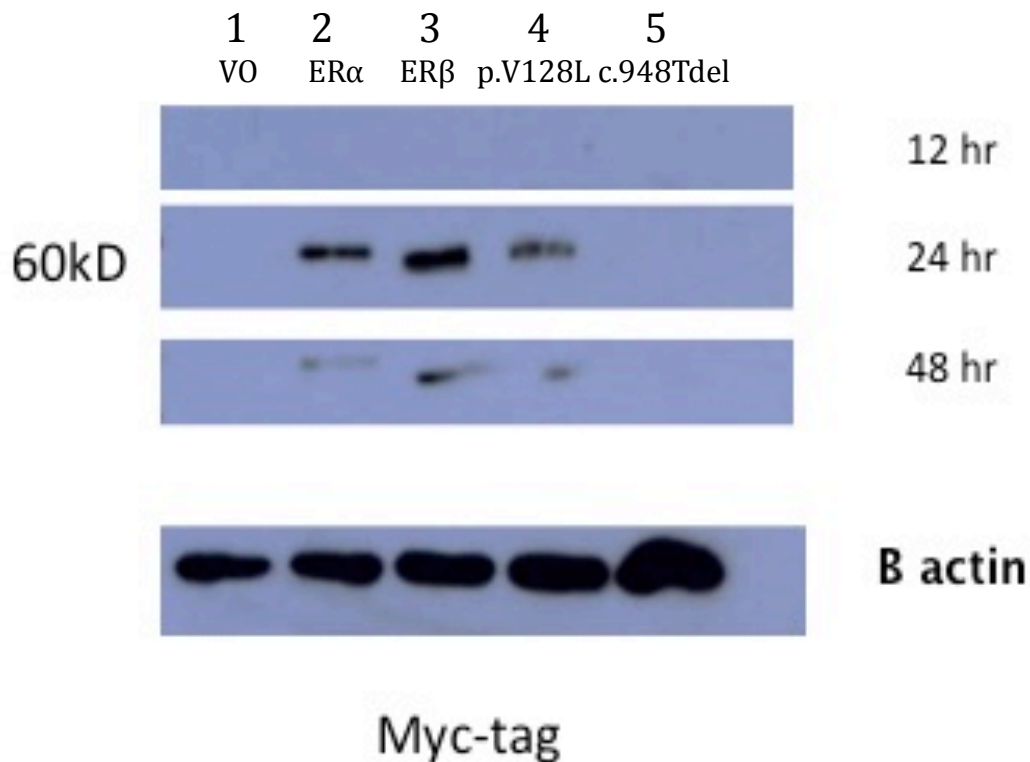


Figure 52. Western blot showing Myc tag primary antibody with secondary mouse conjugated antibody following 12, 24 and 48 hour transient transfection pCMV6-AN Myc tagged vectors (N terminal tagged). In this experiment wild type ER α and wild type ER β and the myc tagged protein products are readily detectable when compared to vector only control (lanes 2 and 3). When transfecting plasmids that are mutated to mimic the identified mutation, p. V128L (lanes 4,) attenuated myc tagged protein product can be seen compared to wild type ER β . When transfecting plasmids that are mutated to mimic the identified mutation c.948delT (lane 5), no myc tagged protein product is detectable (comparable to vector only: lane 1).

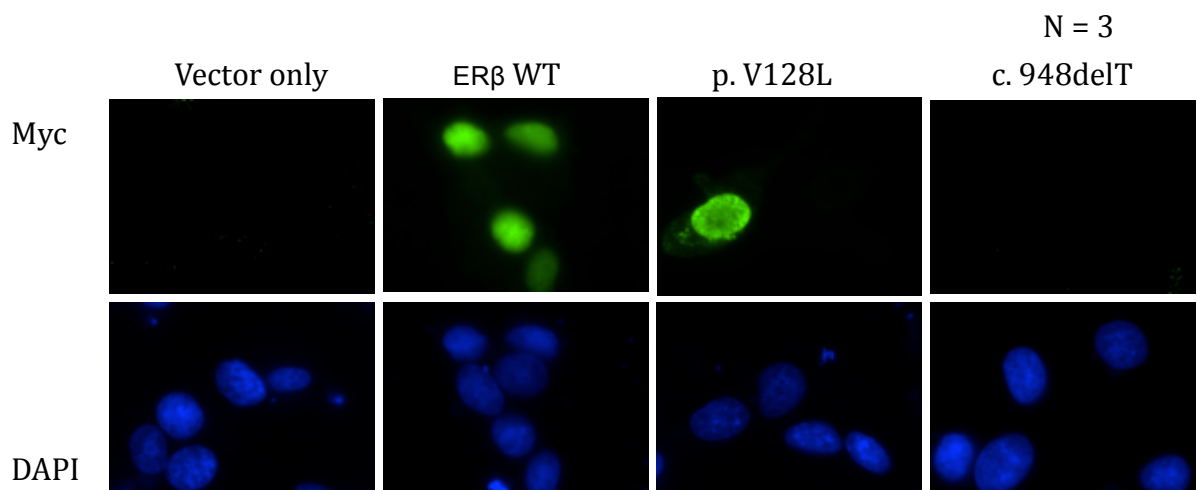


Figure 53. Immunofluorescence. When myc tagged, wild type ER β is transfected, the protein product can be readily detected at the nucleus of successfully transfected cells

compared to vector only (upper panels 1 and 2). The corresponding nucleus of all cells in each field of are shown by DAPI staining (lower panels). When transfecting plasmids that are mutated to mimic the identified mutation, p. V128L, Myc tagged protein product is detected with less frequency than wild type ER β (upper panels 2 and 3). When transfecting plasmids that are mutated to mimic the identified mutation c.948delT, no myc-tagged protein product can be seen.

7.3.3 RET is over expressed in MCF 7 cells transiently transfected with c. 948delT mutants and to a lesser extent p. V128L mutants compared to wild type.

RET protein expression was not detectable in protein extracted from MCF7 or T47D cells in culture (data not shown). RET protein expression was not detected in MCF 7 cells after 24 hour transient transfection with vector only pCMV AN Myc tagged plasmids, or when transfected with different isoforms of wild type ER β . Significant up-regulation of RET was observed with transfection of the ESR2 p.V128L alteration and to a greater extent the ESR2 c. 948delT mutation. Again, maximal expression was demonstrated 24 hours post transient transfection. Figure 55 demonstrates RET expression after 24 hours transient transfection with wild type and mutant ER β . Experiments were reproduced in triplicate.

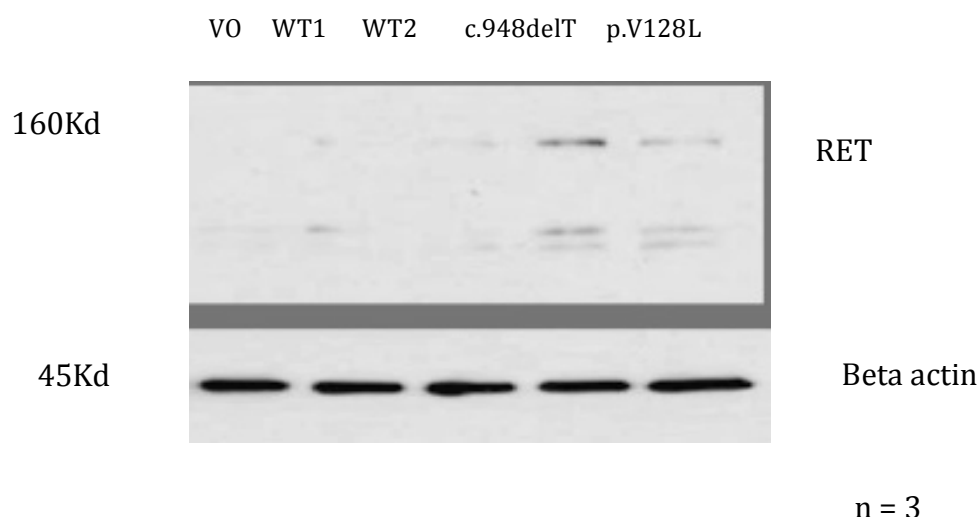


Figure 54. Western blot showing *RET* protein expression in MCF 7 cells after 24 hour transient transfection with pCMV AN Myc tagged plasmids. Here, transfections with wild type *ERβ* in its two most common isoforms (wild type 1 and 2: WT1, WT2), lead to no detectable increase in *RET* protein product. When transfecting plasmids that are mutated to mimic the identified mutation, p. V128L, *RET* protein is more readily detectable than wild type or vector only transfection (lane 5 vs. lanes 2 and 3). When transfecting plasmids that are mutated to mimic the identified mutation, c.948delT, a significant increase in *RET* protein product is seen compared to wild type *ERβ* or vector only transfection (Lane 4 vs. lanes 2 and 3).

7.3.4 *RET* mRNA is overexpressed in MCF7 cells transiently transfected with c. 948delT mutations, in the presence and absence of oestrogen

Following 24 hour transient transfection, MCF cells were treated with either oestrogen or ethanol control. In terms of *RET* mRNA fold change, greatest effects were seen between 24 and 48 hours post oestrogen treatment (data not shown). Significant *RET* mRNA fold changes were demonstrated when transfecting *ERα* treated with oestrogen vs. control (Figure 56). No changes were seen when *ERα* and *ERβ* constructs were co-transfected with or without oestrogen. Co-transfection with *ERα* and *ESR2* c. 948delT mutants showed a 2 fold change in mRNA expression over *ERα* - *ERβ* WT co-transfection. The fold change is consistent with 50% *ERα* alone transfection suggesting loss of the inhibitory effect of WT *ERβ*.

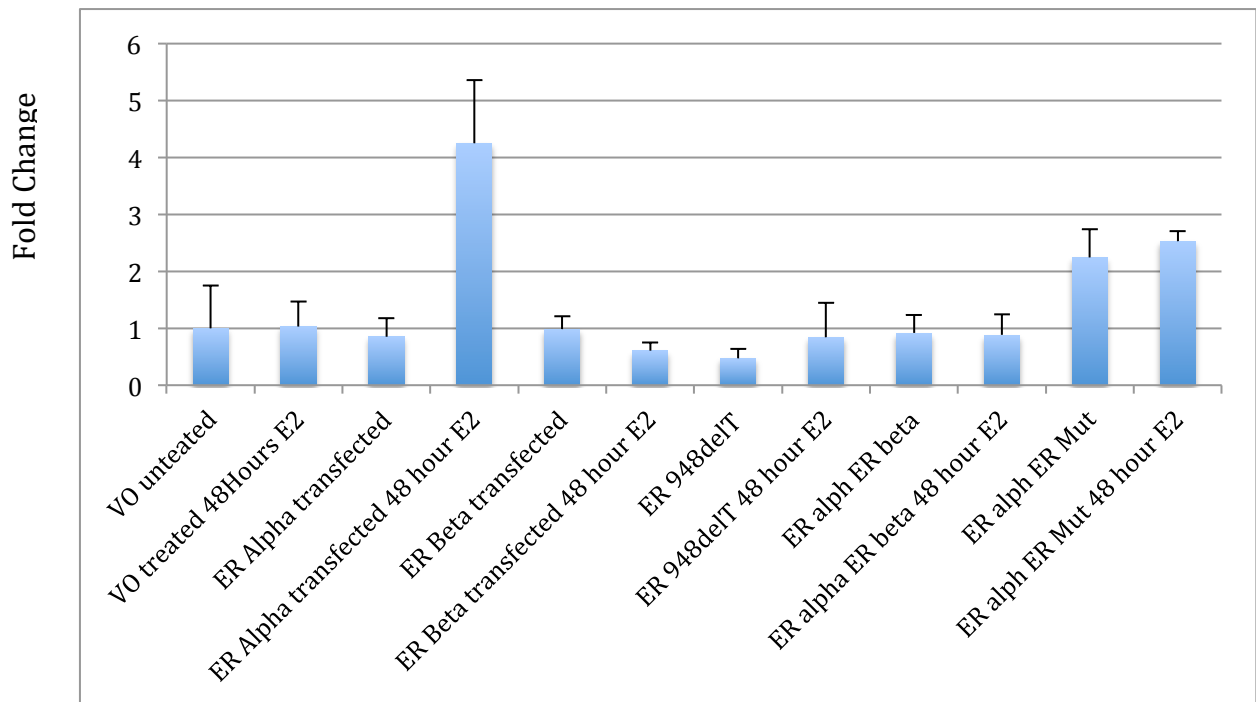


Figure 55. RET mRNA expression in MCF 7 cells initially transiently co-transfected with combinations of AN-myc-tagged plasmids containing, vector only, ER α and ER β WT and the ER β c. 948delT mutant construct. When ER α is transfected alone in the presence of oestrogen, a four fold up regulation of RET mRNA is seen. When ER β is transfected alone or with ER α , no increase in RET mRNA is detected with or without oestrogen suggesting an inhibitory role for ER β . When ER α and ER β c. 948delT are co-transfected there is a two fold increase in RET mRNA production suggesting loss of the inhibitory role of ER β .

7.3.5 Calcitonin staining is not detected in normal thyroid tissue but can be easily seen in CCH and MTC

Calcitonin staining was performed on all members of the index family and all available tumour normal blocks for patients with sporadic disease. Calcitonin staining was not evident in normal thyroid tissue taken from the unaffected side of patients with sporadic disease (0/18 samples). Three offspring of the index family displayed evidence of CCH on a background of normal thyroid

tissue (figure 57, left panel). All cases of sporadic MTC stained avidly calcitonin positive (figure 57, right panel).

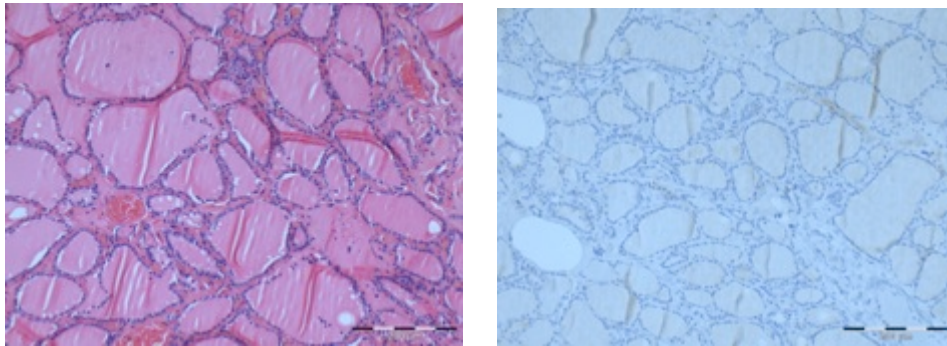


Figure 56. Hematoxylin and Eosin (H and E) staining of normal thyroid tissue showing arrangements of thyrocyte around follicles of thyroglobulin (left panel). Calcitonin staining of normal thyroid tissue taken from the unaffected side of a patient with MTC (right panel).

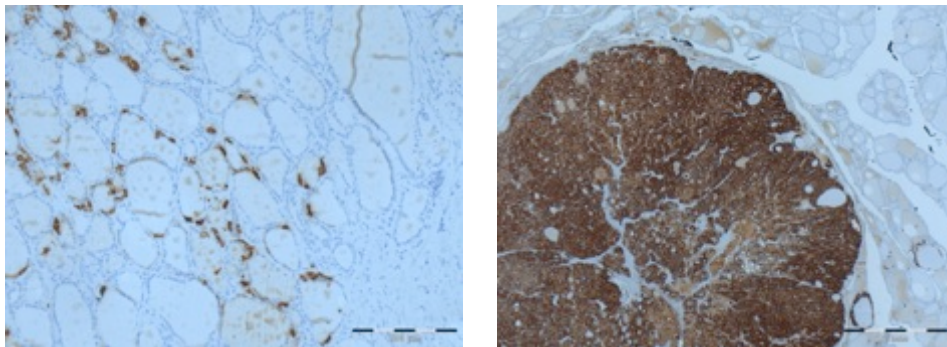


Figure 57. Calcitonin staining in CCH (left panel) and MTC (right panel).

7.3.6 ER β is ubiquitously expressed in normal thyroid tissue

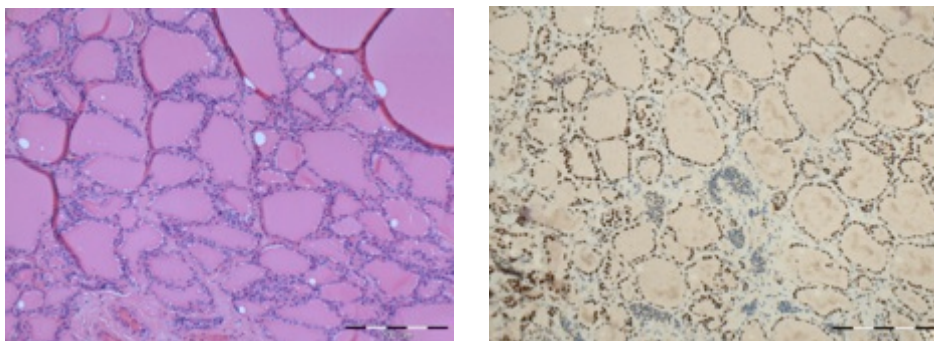


Figure 58. H and E staining of normal thyroid tissue (left) showing thyrocytes (blue staining) arranged in functional units around colloid (pink staining). ER β staining of normal thyroid tissue demonstrating ER β expression in normal thyrocytes.

7.3.7 ER β is lost in tumours in carriers of c. 948delT mutation

ER β expression was seen in 18/18 sporadic MTCs with no germline ESR2 mutation (Figure 61, left panel). By comparison, significant ER β under-expression was observed in MTC tumour carrying germline ESR2 c. 948delT mutation (right panel).

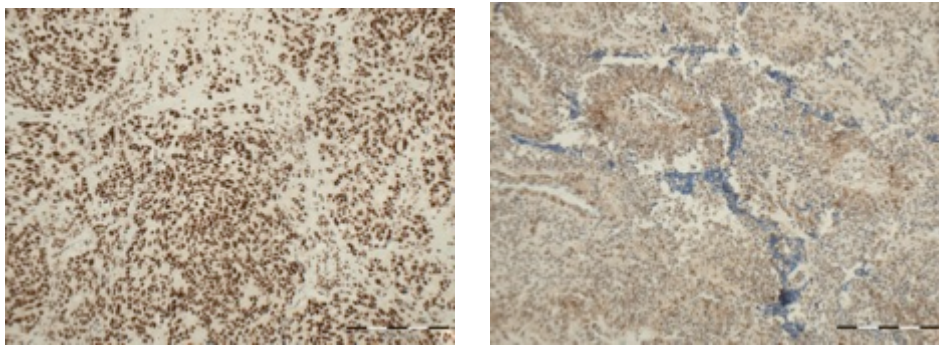


Figure 59. ER β staining in MTC (left) showing dense staining in malignant cells of C cell origin. Right panel from tumour of patient carrying c.948delT mutation showing significant under-expression.

7.3.8 RET is overexpressed in carriers of ESR2 c. 948delT mutation

As part of the initial clinical work-up, RET staining was performed on members of the index family. RET over expression has clearly been identified in C cells and MTC in family members subsequently found to carry the ESR2 c.948delT mutation (Figure 61.) when compared with normal and positive controls (Figure 62.).

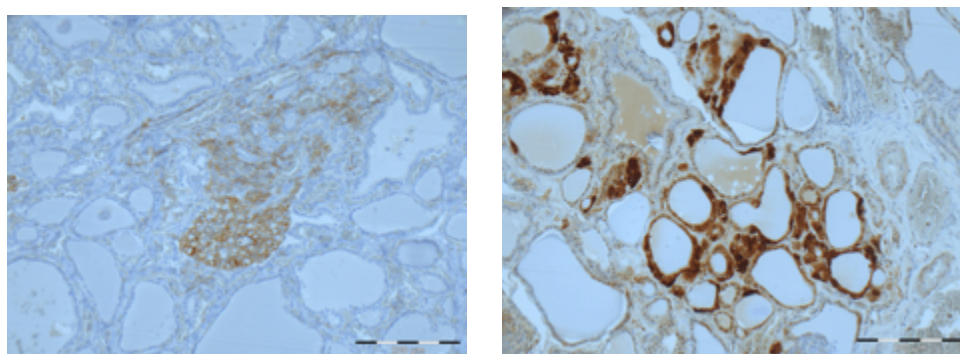


Figure 60. Right panel showing calcitonin staining in familial c.948delT with RET staining (left panel) from the same family member

7.3.9 RET expression could not be determined in MTC or normal thyroid tissue in the wider cohort

RET staining is not routinely performed by the UHB laboratories. RET staining was carried out as per manufacturers guidelines but no staining could be demonstrated in either normal thyroid, CCH or MTC (Figure 62). The positive control (small bowel) as recommended by the manufacturers, shows little positive staining (enteric nervous system only shows positive staining). Multiple antibody titrations were performed without establishing positive staining. The slides have subsequently been sent to a laboratory specialising in RET biology although these data were not available at the time of writing.

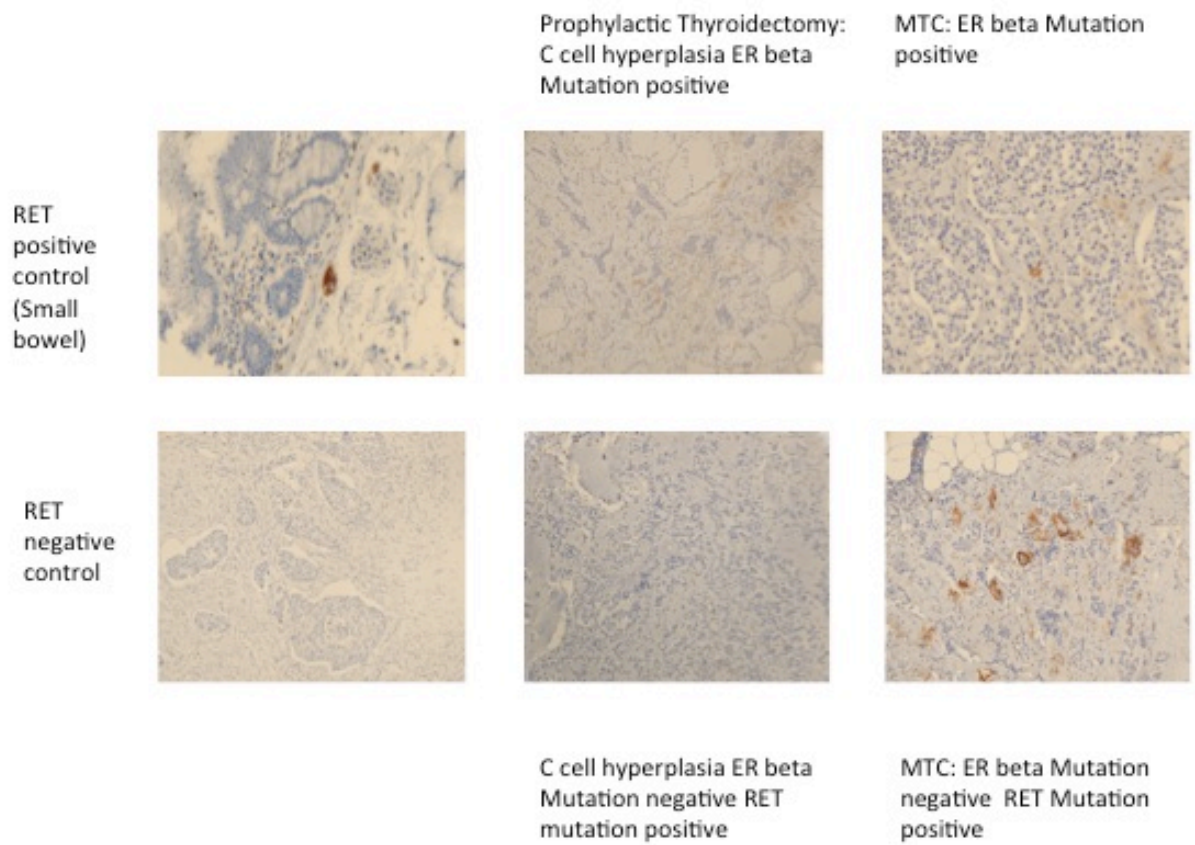


Figure 61. RET staining in positive and negative controls as well as CCH and MTC with and without mutations.

7.4 Discussion

Through protein analysis by western blotting two of the main hypotheses were tested. Firstly, that the c. 948delT mutation produces a null protein and secondly, that transient transfection of the two identified mutations (ESR2 c. 948delT and p.V128L) leads to up regulation of RET protein when compared with wild type transfection and vector only. The question of whether a null or truncated protein would be produced by our deletion is clearly of great importance to the project as a whole as it provides some evidence of the potential interactions that underpin tumour predisposition within the index family. The null protein hypothesis is supported by the western blotting and immunofluorescence results, although these results have to be taken within the context of a single cell line. This is clearly a limitation, and time constraints were the reasoning that evidence from additional cell lines was not sought. Further corroborative work with these issues in mind is currently underway. Further support to the null hypothesis is gained, however, from the immunohistochemistry results. The fact that all normal thyroid and sporadic MTC tumours stain avidly for ER β except for those harbouring the ESR2 c. 948delT mutation is significant, both from a point of adding weight to our null protein hypothesis but in addition, linking *in vitro* findings back to the index family.

If we are to assume that the ESR2 c. 948delT mutation is indeed the single mutation that gives C cells a selective proliferative advantage then it is clearly important to understand the nature of this interaction at the level of the protein. The suggestion from the results presented here is that no protein is

produced or that the protein product is so unstable that it is rapidly degraded by the cellular mechanism of abnormal protein degradation or, more likely based on these data, nonsense mediated mRNA decay (Chang, Imam and Wilkinson, 2007). No demonstrable myc signal was detected when attached to the N terminal end of the protein product of the ESR2 c. 948delT mutant, even at the shortest time interval either by immunofluorescence or by mRNA estimation. Nonsense mediated decay is a cellular mechanism by which abnormal truncated mRNA is removed prior to [transcription](#)^[10] to prevent the potential damaging effects of a truncated protein which may harbour a gain of function (Anczuków et al., 2008). Whether nonsense mediated decay prevents any protein being made, however, is open to debate and has shown to be mutation specific in both BRCA1/2 and TP53 mutations (Anczuków et al., 2008), (Perrin-Vidoz et al., 2002). The possibility remains therefore, that the ESR2 c. 948delT mutation still produces a protein product modified in such a way as to be undetectable by the methods employed here.

The immunohistochemistry results suggests that the model of disease predisposition is one of haplo-insufficiency, whereby expression of the ESR2 c. 948delT mutation in target tissues results in a 50% reduction in the quantity of functional ER β (Figure 61) (Paratore et al., 2002). In the haplo-insufficiency model, WT ER β acts as tumour suppressor gene, possibly by inhibiting the proliferative effects of ER α , and that reducing the dose of its suppressor activity is enough to give tissue harbouring the mutation a selective advantage. Halpo-insufficiency has been implicated in many diseases and goes some way to explaining the additional disease burden not solely

explained by the two hit hypothesis (Knudson et al., 1976)(Meng et al., 1998). As ER β is ubiquitously expressed in human tissues this hypothesis would have to support the notion that C cells or tissues of neural crest origin are uniquely sensitive to the suppressor effect the ER β has on the RET pathway (as other tumours have not been seen to date within the index family). This is of course potentially the case as patients with germline RET mutations (MEN 2 syndromes) only develop tumours in tissues of neural crest origin and not in other tissues (Mulligan et al., 1993).

With regard to ER α and RET immunohistochemistry, no staining was seen in MTC with or without mutations performed as part of this project. It is somewhat surprising that no ER α was seen in any of the examined tumour of normal slides as expression has been variably reported in MTC, normal thyroid tissue and thyroid cancer (Yang, Pearson and Samaan, 1988), (Zeng et al., 2007), (Kumar, Klinge and Goldstein, 2010). To support the hypothesis that proliferation in C cells harbouring the ESR2 c. 948delT mutation was driven by unopposed ER α , it would be expected that up-regulation of ER α would be seen in the index family compared to sporadic MTC tumours. Clearly, as it was not possible to demonstrate ER α expression in any of the examined slides, this is not possible. This could of course indicate that ER α is not up-regulated nor involved in the progression of MTC. However, the ER α antibodies used here have been optimised by the histopathology lab at the QEH as they are widely used as markers for diagnostic purposes in breast cancer where ER α is abundant (as seen in Figure 61. positive control). It is possible that the protocol used was not sensitive enough to detect the lower

levels expected in thyroid tissue. Regarding RET, it is suspected that there may be issues with the supplied antibody (again, not used in current diagnostics) as evidenced by weak staining in the positive control, as it would certainly be expected that RET overexpression in would be demonstrated in some if not all MTC (Eng et al., 1998). Analysis of the historical slides from the index family however, clearly indicates that RET is overexpressed in tumours and C Cells within the thyroid tissue of those harbouring germline ESR2 c.948delT mutation. This is clear evidence that the disease is driven through the RET pathway, and supports the hypothesis that mutations in the oestrogen pathway could lead to RET dysregulation. Further comparative evidence from the wider cohort is needed here and, as discussed, further slides have been sent to another group for verification. Results are awaited (data not available at time of writing).

Chapter 8.

Final conclusions and future directions

8.1 Conclusions

MTC is an aggressive disease with poor outcome and limited treatment options (Kloos et al., 2009). For those with advanced disease, there are few adjuvant treatments and MTC remains primarily a surgical disease (Perros, 2007). As seen in this cohort and in the wider literature, advanced disease at presentation is extremely common (Roman, Lin and Sosa, 2006). Although RET testing is in routine use, the majority (75%) of patients are RET negative and, as such unable to benefit from screening tests (Frank-Raue et al., 2011). There remains a need for better understanding of the genetic and epigenetic events predisposing to the development of MTC. I hope in this project I have gone some way to addressing this need. Although further corroborative evidence is needed, the identification of constitutional ESR2 mutations as MTC predisposing represents a major breakthrough and has potential to translate directly to patients by way of a simple clinical genetic test. With regard to the index family, this has direct implications for future generations. Furthermore, elucidation of the cellular events leading to MTC may also open avenues for targeted drug therapy within both the index family and in the wider sporadic cohort.

8.2 Oestrogens and thyroid cancer

There has been a longstanding link between oestrogens and well-differentiated thyroid cancer, although no specific links to MTC. Clinically, well differentiated thyroid cancers most commonly occur in pre-menopausal women with a lower incidence in men and post-menopausal women (Santin

and Furlanetto, 2011). *In vitro*, oestrogen receptors have been shown to be overexpressed in well differentiated thyroid cancer and proliferation is induced in thyroid cell lines exposed to oestrogen receptor binding ligands (Kumar, Klinge and Goldstein, 2010). Furthermore, ER β has been shown to be overexpressed and ER α underexpressed in both CCH and MTC (Bléchet et al., 2007). Oestrogen receptor positivity, has been proposed as a possible prognostic marker in well differentiated thyroid cancers although to date, these findings have not been translated to routine clinical practice (Magri et al., 2012).

8.3 ER β and ER α

Oestrogens, acting as ligands and binding to oestrogen receptors, are critical in the development of many different tissue types and have been implicated in many human malignancies including cancers of the breast and prostate. Although postulated as important in thyroid disease and thyroid cancer, their exact role is unknown. ER α and ER β are part of the nuclear receptor superfamily of ligand-regulated transcription factors, regulating gene expression through DNA response elements (Zhao et al., 2007). There are classical methods of oestrogen signalling where oestrogen receptors dimerise and bind oestrogen response elements (EREs) that have specific regions for DNA binding and effect chromatin structure, DNA transcription and RNA recruitment. ERs also signal through non-classical, ligand-independent means with similar effects. ER α and ER β are thought to work synergistically and it is the ratio of their expression that determines the overall effect on cellular transcription. With the discovery of EREs in the RET promoter pathway, it

follows that, a major disruption in the structure of ER β , such as could be caused by a ESR2 c. 948delT mutation, could lead to a significant alteration in the transcriptional properties of the cell and therefore predispose to malignant change. This hypothesis is supported by the functional work and immunohistochemical analysis presented here.

8.4 Sporadic MTC

Conformation of abnormalities of the ESR2 gene in somatically derived DNA, represents a significant finding and potentially opens new avenues in understanding of MTC and its pathogenesis. In the future, it may open potential for new therapeutic targets. If the mutated ER β protein acts by exerting a dominant negative effect, that is, forms a non-functioning protein that does not inhibit ER α , it follows that drugs which inhibit ER α may reduce the proliferative effects associated with oestrogen stimulation. ER α is prevalent in many breast cancers and the sensitivity of breast cancers to oestrogen is a major marker of prognosis, in part due to the presence of the oestrogen receptor as a marker of differentiation, but also for its use as a drug target. Selective oestrogen receptor antagonists (SERMS), such as tamoxifen have been used successfully to treat oestrogen sensitive breast cancers for many years. If some thyroid cancers are oestrogen sensitive then it may be that such therapies may be useful in the treatment of MTC or well differentiated thyroid cancer. Indeed, tamoxifen has been shown to inhibit cellular proliferation in MTC cancer cell lines (Yang, Pearson and Samaan, 1988) (Weber et al., 1990).

8.5 Limitations

As with all studies of rare disease, access to cohorts of patients and tissue for analysis is always challenging. Although there were significant successes in recruitment, this project is still relatively small, particularly when performing analysis sub groups such as RET negative families and sporadic disease with young onset. These issues were always going to be potentially problematic and are particular to inception studies such as this, rather than adjuncts to larger studies where significant numbers of patients have already been recruited. The main effect in a small cohort size such as this is the confidence with which identified mutations can be taken forward to functional studies. We would obviously have liked to have more certainty at the start of the project regarding the suitability of ESR2 c. 948delT as a disease causing mutation rather than an incidental finding by, for example, identifying convincing ESR2 mutations in other families. It is obviously a big step both financially and with regard to time investment to begin functional work. The other issue faced here with an inception project is ultimately the amount of time and resources spent on the gathering of data and recruitment and the knock on effect to the functional work towards the end of the project. With further funding secured to take the project forwards, we hope that the groundwork has been completed and the project will gain future successes.

It is possible still that candidate mutations other than ESR2 c. 948delT identified by WES were MTC predisposing. Alternatively, and I believe more likely, is that this is a unique finding within this family. Indeed, genome wide association studies suggest that there is a significant proportion of heritable

disease not accounted for and rare or unique mutations may go some way to explaining this disparity (Ng et al., 2009). The functional work presented here, however, does support the hypotheses that the correct mutation was identified. It may be that either through wider recruitment or through collaboration with other teams it is possible to confirm the uniqueness or otherwise of this mutation in familial non-RET MTC. Ultimately the on-going functional work will unpick the relationships with more certainty.

The functional work shows that mutations of the ESR2 gene lead to dysregulation of the oestrogen-signalling pathway and ultimately to RET pathway up-regulation and a cellular proliferative advantage. The aim was to show this effect in as many possible ways and proposals for future experiments are outlined in the following section. Further evidence from these additional proposed experiments should strengthen our hypotheses prior to moving forward to treatment-based experiments.

8.6 Future directions

8.6.1 ESR2 mutations as diagnostic test

Detection of the ESR2 c. 948delT mutation could form part of the diagnostic work up for patients with non-RET MTC. This of course would require acceptance and peer review of this work, and this is currently underway. It would be expected however that, based on our tested cohort and mutation databases, constitutional ESR2 mutations are rare and only likely to account for small numbers of patients overall with MTC.

8.6.2 Further exome sequencing using the existing cohort and newly recruited families

The findings presented here support the theory that much of the heritable disease seen in clinical scenarios is explained by mutations with low frequency, possibly in single families. As demonstrated, exome sequencing with reference back to the patient specific data and to a larger cohort of patient may be the ideal tool for this. Using the additional families identified here as a starting point for further exome sequence analysis is warranted. Having established a method that has generated results and having reference cohort to refer to, will significantly reduce the time and effort required to invest in this in the future.

8.6.3 Examination of ER β isoforms

Some of the complexity encountered in this project may be explainable^[11] by the nature and function of the isoform of ER β and the relative paucity of data to distinguish specific roles of each individual isoform. Ultimately, we do not have complete knowledge of the differing role and to what extent function of each specific isoform is modulated by local factors in the environment.

8.6.4 Sequencing of ESR1

Although it is postulated that it was the interaction between ER α and ER β that most likely would explain up-regulation of the RET pathway in this family, we have not been able to show that definitively, either in the staining characteristics of the immunohistochemical slides, or in the mRNA studies. There are many possible reasons for this, as explained in Chapter 7. It would be interesting to see whether germline or somatic mutations in ESR 1 play a role in MTC predisposition, but further sequencing was beyond the scope of this study.

8.6.5 Verification of null protein hypothesis in further cell lines and knockdown with siRNA

Proving that the same mechanism of RET pathway up regulation and cellular proliferation can be induced by multiple models is important and, although beyond the scope of the work presented here, will be a major feature of the on-going work on this project and form part of the presentation for peer

review. Establishing that a siRNA knock down model of ER β leads to up-regulation and cellular proliferation would add weight to the results already obtained that the null protein model that we predict from the ESR2 c. 948delT mutation leads to proliferation.

8.6.6 Verification of downstream activators other than RET

The hypothesis, based on the presentation and phenotype of the family is that the mutation predisposing to disease is most likely to lead to up-regulation of the existing pathway, and indirectly influence the expression of RET via another mechanism. The ER pathway, with upstream binding to the RET gene is a perfect example of this. It remains possible, however, that mutations of ESR2 lead to cellular proliferation and differentiation through RET independent pathways, and that examination of downstream effectors other than RET would aid in understanding of the mechanics of cancer predisposition. Again, this will form a part of the on-going studies.

8.7 Overall conclusion

I believe that this project has achieved three outcomes. Firstly, through project advertisement, presentations and the establishment of collaborative teams both within and without the UK, I have raised awareness of some of the issues within the field of MTC, both in familial and the often overlooked sporadic disease, stimulating interest in future research and collaboration. Establishing a cohort of patients with sporadic disease has aided directly in this project, but will also hopefully serve as resource reservoir for future projects. The additional families recruited towards the end of the project will enable expansion of the research, and has already attracted further funding.

Secondly, using next generation sequencing techniques, I have established a method for novel cancer gene mutation discovery in the context of MTC, something not achieved since the discovery of the disease causing RET gene mutations in MEN 2a/b in the early 1990s (Eng et al., 1995) (Eng et al., 1994). Although WES has been used in the context of MTC, no non-RET mutations have been identified to date and as such no novel pathways that might be exploited therapeutically (Qi et al., 2011).

Finally, by identifying ESR2 mutations as potentially predisposing in familial MTC and sporadic disease, I have provided evidence that disease predisposition is driven through up-regulation of the RET pathway. Further work is required to fully unpick the molecular pathways here but we believe this project provides a platform on which to base future studies.

Chapter 9.

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Appendix 1.

Molecular genetic investigation of familial non-RET medullary thyroid carcinoma (MTC)

Smith JA, Maher ER, Woodward ER, Watkinson JC. Institute of Biomedical Research, University of Birmingham

Background

Medullary Thyroid Cancer MTC is an aggressive disease associated with poor outcomes

It accounts for between 5 and 10% of thyroid cancer in the UK

Survival is linked to disease stage at presentation

As a rare cancer both the British and American Thyroid Associations recommend management in multi disciplinary teams

25% of MTC is accounted for by MEN 2 with RET mutation

With routine RET testing there are a significant proportion of apparently familial MTC who test RET negative

These individuals could harbour other genetic abnormalities as yet undetermined that could also have significant implication for apparently sporadic cases of MTC

Aims

To identify novel MTC predisposing genes and to assess their contribution to familial and sporadic MTC tumourogenesis.

Methods

Using our identified RET negative pedigree we will perform:

whole exome sequencing to identify candidate genes for further investigation

copy number array analysis to exclude genomic deletions/amplifications

Recruitment of further RET negative MTC families both nationally and internationally to verify our results

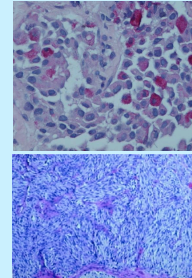


Figure 1/2. Histological preparations demonstrating medullary thyroid cancer



Figure 3. Total thyroidectomy specimen medullary thyroid cancer

We need your help with recruitment!

We are looking for: RET negative-

Apparently sporadic cases of MTC developing before the age of 40

Any familial case of C cell hyperplasia/MTC

RET positive /negative fresh frozen MTC samples (ideally) or paraffin blocks

What will happen to enrolled patients:

Patients consenting to be involved in the study will have blood taken for RET testing

Pathological specimens will be requested for re examination

Contact tracing may take place

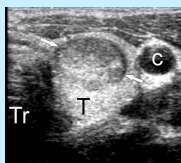


Figure 4/5 Ultrasound and computed tomographic images of MTC

How to refer patients/for more information regarding the study

Please refer any suitable patient using a standard hospital letter to Joel Smith C/O Emma Woodward at:

Clinical Genetics Unit
Birmingham Women's NHS Foundation Trust
Mindelsohn Way,
Edgbaston, Birmingham B15 2TG

Email: e.r.woodward@bham.ac.uk
Tel: 01216272630

Contributors

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