Effects of subclinical hypothyroidism on bone mineral density and cardiometabolic risk: genetic and immune influences

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CONTENTS

SUMMARY

Effects of subclinical hypothyroidism on bone mineral density and cardiometabolic-risk: genetic and immune influences

Subclinical hypothyroidism (SH) affects 3-8% of the population and is associated with hypertension, dyslipidaemia and altered bone mineral density (BMD). Metabolic anomalies in thyroid disease have been attributed to thyroid hormone variation but mice deficient in thyrotropin (TSH) receptor (TSHR) have low BMD, despite normal thyroid hormones, suggesting that TSH/TSHR function in bone is important. SH has several aetiologies including thyroid autoimmunity characterised by thyroid peroxidase autoantibodies (TPO-Ab), inactivating TSHR mutations (TSHR-M) and FOXE1 polyalanine tract length (FOXE1-PTL) polymorphisms. I hypothesise differential bone effects in SH relating to these causes. Similarly the effects of SH on metabolic outcomes is unclear and may also depend on SH aetiology.

Principle aims -

1. To determine the prevalence of heterozygous TSHR-Ms in SH.
2. To evaluate body composition and metabolic parameters according to i) TSH, T4 and T3; ii) TPO-Ab; iii) TSHR-M or polymorphism; iv) FOXE1-PTL polymorphism.

156 women and 52 men, mean age 51 with primary untreated SH were recruited. Blood samples were obtained for biochemistry (thyroid & lipid profiles, TPO-Ab, HOMA-IR) and blood pressure (BP) and anthropometric data collected. TSHR and FOXE1 were genotyped. Dual-energy X-ray absorptiometry generated Z-scores. Stepwise multivariate regression analyses were performed.

Half of the cohort had TPO-Ab; 6% had TSHR-Ms (essentially TPO-Ab negative) and 60% expressed FOXE1$^{14/14}$-PTL. TSH and TPO-Ab associated negatively with BMD-Z at lumbar spine but not hip whereas free-T3 and male gender associated negatively at both sites. TSHR-M status did not influence BMD-Z despite lower free-T3 relative to TSH.

Free-T3 associated positively with BP and HOMA-IR. FOXE1-PTL$^{14/14}$ associated positively with free-T3 and negatively with BP. TSH, TSHR-M and TPO-Ab status showed no metabolic associations but unexpectedly TSH showed a positive association with T3.

SH is considered homogenous but my results illustrate its heterogeneity and highlight the need for studies accounting for aetiology in order to optimise clinical management.

Dedication

I dedicate the thesis firstly to my husband Paul Mortimore and thank him for his patience, self-compromise and support. Secondly this is dedicated to the memory of our first born child, a son, born at 21 weeks gestation on the 7th of September 2014.

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PUBLICATIONS AND PRESENTATIONS

Publication arising from this research:

de Lloyd A, Bursell J, Gregory JW, Rees DA, Ludgate M. TSH receptor activation and body composition. Journal of Endocrinology. 2010 Jan; 204(1):13-20.

International Meetings:

2015 European Congress of Endocrinology (ECE), Dublin, Ireland.

- TSH and free-T3 correlate negatively and independently with bone mineral density in adults with subclinical hypothyroidism. Endocrine Abstract; 37 EP253
  1st author; poster presentation

- Adverse metabolic correlations relate to free-T3 levels in subclinical hypothyroidism; common FOXE1 polymorphisms associate with blood pressure. Endocrine Abstract; 37 GP26.03
  1st author; poster presentation

2014 European Thyroid Association (ETA), Santiago de Compostela, Spain.

- An intronic deletion in phospholipase-C B1 (PLCB1) associated with euthyroid multinodular goitre (MNG) and risk of progressing to papillary thyroid cancer (PTC).
  5th author; oral presentation by 1st author

2013 European Thyroid Association (ETA), Leiden, The Netherlands.

- Evaluating the prevalence of thyrotrophin receptor (TSHR) inactivating mutations in adults with subclinical hypothyroidism (SH); impact on bone density. OP66
  1st author; oral presentation in the main auditorium.

2013 British Thyroid Association (BTA), London, UK

- Exploring variation in bone density in a well characterised and genetically profiled Subclinical Hypothyroid (SH) adult population in South Wales. P10
  1st author; poster presentation

2013 British Endocrine Society (BES), Harrogate, UK

- Development of an inductively coupled plasma-mass spectrometry method for measurement of urine iodine and assessment of iodine status in subclinical hypothyroidism. Endocrine Abstracts (2013) 31 P38.
  3rd author; poster presentation

2011 British Endocrine Society (BES)

- Impact of TSHR signalling on bone density and body composition.
  1st author; poster presentation

2010 British Endocrine Society (BES), Manchester, UK

- Impact of extra cellular matrix changes on adipogenesis. Endocrine Abstracts (2010) 21 P156.
  3rd author; poster presentation

2010 International Thyroid Congress (ITC), Paris, France

- FOXE1 polyalanine tract variants; Impact on transcriptional activity and thyroid status.
  P-0819
  4th author; poster presentation

National and local meetings:

2012 Welsh Endocrine and Diabetes Society Meeting (WEDS)

- The molecular characterisation of subclinical hypothyroidism
  1st author; oral and prize presentation

2012 Postgraduate Research Day, Cardiff University, UK

- Thyrotrophin receptor activation and its effect on body composition
  1st author; poster presentation

2010 Postgraduate Research Day, Cardiff University, UK

- Impact of TSH receptor signalling on bone density and body composition in a subclinical hypothyroid population
  1st author; poster presentation

2009 Postgraduate Research Day, Cardiff University, UK

- Effect of a single functional TSH receptor on bone metabolism and body composition
  1st author; poster presentation

Abbreviations

AF	Atrial Fibrillation
ALP	Alkaline Phosphatase
AR	Autosomal recessive
ATD	Autoimmune thyroid disease
BAT	Brown adipose tissue (brown fat)
BF	Body fat (used in %BF)
BMI	Body Mass Index
BMPs	Bone morphogenic proteins
BP	Blood pressure
bp	Base pair
BT	Bone turnover
BTM	Bone turnover markers
Ca^{2+}	Calcium or corrected calcium
C.I	Confidence interval
CH	Congenital hypothyroidism
CMIA	Chemiluminescent microparticle immunoassay
CRF	Clinical research facility
CTX	C-terminal telopeptide of type 1 collagen
CVD	Cerebrovascular disease
DI	Iodothyronine deiodinase enzymes
dHPLC	Denaturing high performance liquid chromatography
DXA	Dual-energy X-ray absorptiometry
EB	Ethidium bromide
ESC	Embryonic stem cell
f-glucose	Fasting glucose
FH	Family history
FN	Femoral neck
Frz	Secreted frizzled related proteins
FSH	Follicle stimulating hormone
FTH	Free thyroid hormone

GD	Graves' disease
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GH	Growth hormone
GPCR	G-protein coupled receptor
GO	Graves' orbitopathy
GWAS	Genome Wide Association Study
hCG	Human Chorionic Gonadotrophin
HPT	Hypothalamic-pituitary-thyroid axis
HDL	High density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
HSCs	Haemopoetic stem cells
ICP-MS	Inductively coupled plasma mass spectrometer
IGF-1	Insulin-like growth factor-1
IHD	Ischaemic heart disease
IP_3	Inositol triphosphate
L1	1^{st} lumbar vertebra
L2	2^{st} lumbar vertebra
L3	3^{st} lumbar vertebra
L4	4^{st} lumbar vertebra
LH	Luteinising Hormone
LDL	Low density lipoprotein cholesterol
LFTs	Liver function tests
LS	Lumbar spine
mRNA	messenger RNA
MRA	Multiple regression analysis
MSC	Mesenchymal stem cell
MVHR	Multivariable-adjusted hazard ratio
N (or n)	Number
Na/I	Sodium Iodide (used in Na/I symporter)
NTD	Nodular thyroid disease
P1NP	Procollagen type 1 N-terminal propeptide

PCR	Polymerase chain reaction
PH	Primary hypothyroidism
PKA	Protein kinase A
PKC	Protein kinase C
Pm	Polymorphism
PO^{4-}	Phosphate
PTH	Parathyroid hormone
PTL	Polyalanine Tract length
R	Regression coefficient
RANK(L)	Receptor activator of nuclear factor κβ (Ligand)
rhTSH	Recombinant TSH
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SV	Sequence variant
ScTD	Subclinical thyroid disease
SD	Standard deviation
SE	Standard error
SH	Subclinical hypothyroidism
Shyper	Subclinical hyperthyroidism
T3	free triiodothyronine
T4	free tetraiodothyronine (thyroxine)
TC	Total cholesterol
Tg	Triglycerides
TG	Thyroid gland
TH	Total hip
TLS	Total lumbar spine
TFT	Thyroid function test
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TPO	Thyroid peroxidase
TPO+ (or TPO+ve)	Thyroid peroxidase antibody positivity
TR	Thyroid hormone receptor

TRT	Thyroxine replacement therapy
TRH	Thyrotrophin Releasing Hormone
TSH	Thyroid Stimulating Hormone
TSHR	Thyroid Stimulating Hormone Receptor
TSHR*	Constitutionally active TSHR
TSHR-M	Thyroid Stimulating Hormone receptor mutation
TSI	Thyroid Stimulating Immunoglobulin
TTF	Thyroid transcription factor
UHW	University Hospital of Wales
UCP1	Uncoupling protein 1
VSMC	Vascular smooth muscle cells
VEGF	Vascular endothelial growth factors
WHO	World Health Organisation
WHR	Waist to hip ratio
WT	Wild type

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CHAPTER 1: INTRODUCTION

Subclinical Hypothyroidism (SH) was first described as a specific entity by Hall and Evered (1973). SH describes a biochemical condition consisting of an elevated thyrotrophin hormone (TSH) with normal free thyroid hormones (FTH) levels. As its name suggests this may be "subclinical" and not associated with specific symptoms. SH may be considered a compensatory state where a raised TSH overcomes a degree of resistance in the system enabling maintenance of normal FTH levels. This is best explained by understanding the Hypothalamic-Pituitary-Thyroid (HPT) axis.

1.1 Hypothalamic-Pituitary-Thyroid axis

The HPT axis describes the endocrine system which maintains and regulates FTH homeostasis (Reichlin 1967). Thyrotrophin releasing hormone (TRH) is a peptide hormone synthesised in the paraventricular nucleus in the hypothalamus (located just above the brain stem and below the thalamus (Rasmussen 1938). TRH passes down the hypophyseal portal tract (vasculature) to the anterior pituitary gland (located below the hypothalamus) where it stimulates the release of TSH from the pituitary thyrotrophs (Sonenberg 1958). TSH circulates to the thyroid gland (TG) where it interacts with the TSH receptor (TSHR) on thyroid follicular cells to stimulate the release of thyroid hormones: Tetraiodothyronine (T4 or Thyroxine) and Triiodothyronine (T3). The thyroid hormones negatively feedback on the hypothalamus and pituitary gland (Scanlon *et al* 1978) to regulate FTH levels (see figures 1.1 & 1.2).

Free thyroid hormone refers to the bioavailable (un-protein bound) and active hormone in serum. It makes up <1% of the total circulating pool (Winter & Signorino 2001; Boelaert & Franklyn 2005) and feeds back on the HPT axis. For example, if T4 levels fall, negative feedback on the hypothalamus and pituitary falls, enabling TRH and TSH levels to rise to increase thyroid hormone synthesis. Whereas high FTHs have the opposite effect, serving to reduce thyroid hormone production. Thus in health, the HPT axis tightly regulates FTH levels.

Figure 1.1: Schematic illustration of the anatomical location of the Hypothalamus, Pituitary and Thyroid Gland relative to a photograph of the author

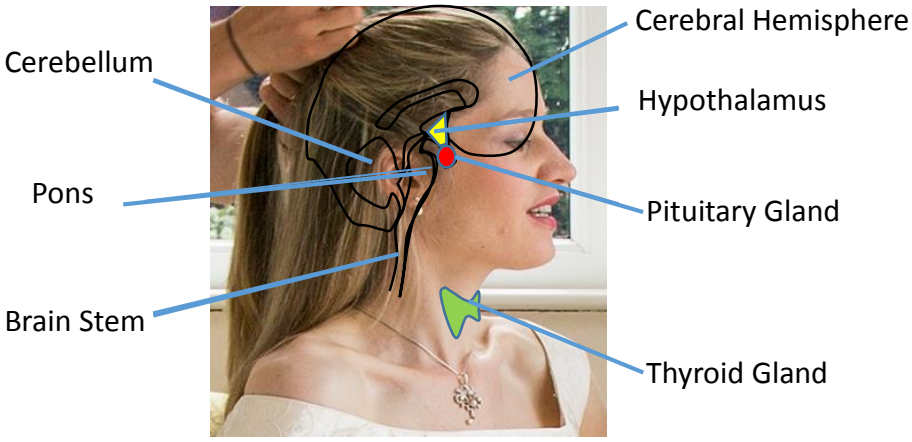
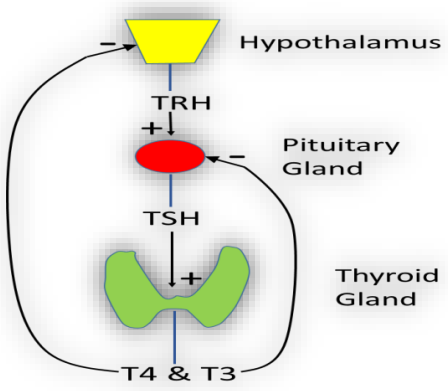


Figure 1.2: Negative feedback regulation of the Hypothalamic-Pituitary-Thyroid axis



Figures 1.1 & 1.2 depict the hypothalamus in yellow, the pituitary in red and the thyroid in green. Arrow heads have adjacent symbols indicating stimulation (+) or suppression (-) within the axis

1.2 Thyroid Gland

The TG is present in all vertebrates and is responsible for the synthesis of thyroid hormones (Lynn & Wachowski 1951, Dickhoff & Darling 1983). The development of a specialised gland with the ability to concentrate iodide and synthesise thyroid hormone may have been an important evolutionary step enabling higher life forms to evolve from the iodine rich ocean to the iodine deplete waters and lands (Venturi *et al* 2000). The endostyle, a ciliated groove

in the ventral pharynx of simpler life forms may represent the primitive origins of the TG (Lynn & Wachowski 1951).

The TG was named, and its anatomy described in detail by Thomas Wharton (1656). The gland is located anteriorly in the lower neck just below the cricoid cartilage. It has two lobes (connected by an isthmus) that drape around the larynx to the oesophagus posteriorly. Embryologically it develops from an outpouching of the immature pharynx (the foramen caecum at the base of the tongue marks this point) and descends via the thyroglossal duct to this location (Zapanta & Shokri 2014). A human foetus aged 18-20 weeks has an immature but functional HPT axis and is able to synthesis thyroxine (Morreale de Escobar *et al* 2000). Up until 12 weeks gestation a human foetus is completely dependent on the transfer of maternal thyroxine across the placenta for normal development.

The TG is composed of a mass of follicles (Hertzler 1939), roughly spherical in shape containing a colloid known as thyroglobulin. The follicles alter in size in response to metabolic demand (reflecting a change in follicular cell number, and size). The follicles are encased by a layer of cells known as follicular cells which lie in proximity to surrounding fenestrated capillaries. Scattered between the follicles are parafollicular C-cells responsible for the production of calcitonin (Pearce 1966). C-cells are functionally and embryologically distinct from the tissues that synthesise thyroid hormone. The C-cells originate from neural crest (Pearce & Polak 1971) and calcitonin has been considered a neuro-peptide (Zaidi *et al* 1987). Although calcitonin is considered functionally redundant in humans, it lowers serum calcium and contributes to calcium homeostasis in lower mammals.

Thyroid follicular cells synthesise and secrete thyroid hormones in response to TSH-TSHR engagement. This precipitates a series of physiological events simplified and summarised below: -

1. Iodide is *trapped*; actively transported from blood to thyroid follicular cells via the sodium iodide (Na/I) symporter on the plasma membrane. Pendrin (a protein transporter) may enable the efflux of iodide from the follicular cells to the thyroid follicle for organification (Bizhanova & Kopp 2009).
2. Thyroid follicles contain thyroglobulin, a tyrosine rich glycoprotein.

3. Thyroperoxidase (TPO) is an enzyme on the apical surface of follicular cells that oxidises iodide to iodine (in the presence of hydrogen peroxide; generated by the enzyme DUOX2). TPO covalently binds iodine to tyrosine in thyroglobulin.
4. TPO generates T4 and T3 by combining diiodothyronines or monoiodothyronines (diiodothyronine + diiodothyronine = T4, monoiodothyronine + diiodothyronine (in appropriate combination) = T3). Thyroid hormones remain complexed in thyroglobulin.
5. Follicular cells endocytose mature thyroglobulin which undergoes lysosome digestion to liberate thyroid hormones.
6. Thyroid hormones diffuse into surrounding capillaries and on to the general circulation. (adapted from *Boron 2003*)

1.3 Thyroid Status

Thyroid status is a term used to describe the relative amounts of FTHs in an individual termed; 'hyperthyroid', 'subclinical hyperthyroid' (Shyper), 'euthyroid', 'subclinical hypothyroid' (SH) and 'hypothyroid'. Hyperthyroidism describes an excess of FTHs; Shyper describes a suppressed TSH with normal FTHs; euthyroidism describes *normal* TSH and FTHs; SH describes a raised TSH with normal FTHs, and hypothyroidism describes subnormal FTH levels.

Thyroid Function Tests (TFTs) detail an individual's TSH and T4 values +/- T3 (at the time the sample was taken) and enable the cause of abnormal TFTs to be determined in most cases. As such, thyroid disorders are further categorised according to the gland within the HPT axis that is failing to function normally. In this context *primary* refers to the abnormality occurring in the TG, *secondary* to the abnormality occurring in the pituitary, and *tertiary* to the abnormality occurring in the hypothalamus. However, given that TFTs do not differentiate secondary from tertiary causes (as TRH is not included in TFTs) these are collectively referred to as *central* hypo or hyperthyroidism (Freeman *et al* 2009). These TFT classification categories are summarised in table 1.1 below.

Table 1.1: Thyroid status and determinants of primary, secondary and tertiary thyroid dysfunction

Thyroid Status (below)	**Free T4**	**TSH**	**TRH**	**HPT axis category**
Hypothyroidism	↓	↑ ↓/ normal ↓	↑ ↑ ↓	primary secondary tertiary
Subclinical hypothyroidism	Normal	↑	↑	primary
Euthyroid	Normal	normal	normal	no abnormalities
Subclinical hyperthyroidism	Normal	↓	↓	primary
Hyperthyroidism	↑	↓ ↑/normal ↑	↓ ↓ ↑	primary secondary tertiary

The arrows indicate: subnormal (↓) or supra-normal (↑) hormone levels. HPT represents the Hypothalamic-pituitary-thyroid axis

1.4 Thyroid hormones, receptors and the deiodinase enzymes

Thyroid hormones are ubiquitous and act on almost all cells in vertebrates (Eales 1997, Hulbert 2000). They are key determinants of the *metabolic rate* (carbohydrate, protein, lipid metabolism and temperature regulation) (Magnus-Levy 1895, Thomas 1957, Tata *et al* 1962). They are necessary for healthy reproductive function (Dittrich *et al* 2011), and growth and development (Khamsi & Eayrs 1966, Tarim 2011). Their influence on the growth hormone (GH) - insulin-like growth factor-1 (IGF-1) axis is recognised (hypothyroidism is associated with diminished GH/ IGF-1 secretion (Hervás *et al* 1976, Chernausek *et al* 1983, Iglesias *et al* 2001)).

T3 (rather than T4) is the active cellular hormone, being markedly more potent than T4 (Gross & Pitt-Rivers 1953). The binding affinity of T3 for thyroid hormone receptors (TR) is approximately (~) 15 times greater than T4 (Lin *et al* 1990). In health, ~80% of the hormone released from the TG is T4, and ~20% T3 (Kansagra *et al* 2010). T4 is often considered a

'prehormone' as it can be converted to T3 by the iodothyronine deiodinase enzymes (DI). Indeed ~80% of T3 utilised by cells is generated by tissue specific DI (Irizarry *et al* 2014).

The DI are selenoenzymes that provide additional defence against abnormal thyroid hormone levels at the tissue, cellular and intracellular level (Köhrle 1996). For example; when FTH levels fall D2 DI is upregulated (to increase T3) and D3 DI downregulated (to reduce inactivation of T3) (see table 1.2). The reverse occurs with excess FTH, in an attempt to normalise T3 (Salvatore 2011). The expression of tissue Dis, during development, varies in an age- and stage-dependent manner (St Germain *et al* 2009) thus enabling control of T3-mediated cellular differentiation, growth and metabolism. A variety of non-thyroidal endogenous signalling molecules also affect the function of DIs (Gereben *et al* 2008).

Table 1.2: The D1, D2 and D3 iodothyronine deiodinase enzymes

Iodothyronine deiodinase enzyme and gene name	Location	Function
D1 (Type 1) *DI01*	Plasma membrane; Thyroid Liver Kidney Brown fat Skeletal muscle	Converts T4 to T3 (major source of circulating T3)
D2 (Type 2) *DI02*	Endoplasmic Reticulum; Thyroid Pituitary Hypothalamus Brain Brown fat Skeletal muscle	Converts T4 to T3 (local T3 production)
D3 (Type 3) *DI03*	Plasma membrane ; CNS Placenta Pregnant uterus Embryonic tissues Rarely malignancies	Inactivates T4 & T3 :- -T4 to reverse T3 -T3 to 3,3'-T2

(adapted from Wassen 2005)

Thyroid hormone receptors (TR) are nuclear receptors that modify gene expression. Although the uptake of lipophilic thyroid hormones was thought to be a passive process, we now know that this is not the case. Thyroid hormone transporters include the monocarboxylate

transporters; MCT-8 and MCT-10, OATP (Organic Anion Transporting Polypeptide) and the amino acid transporters LAT-1 and LAT-2 (Kinne *et al* 2011). MCT-8 is the most specific and well-studied of these transporters and its functional absence in the Allan-Herndon-Dudley syndrome demonstrates its importance. This is an X-linked recessive disorder resulting from mutations in *MCT8* located on Xq13.2 (Schwartz *et al* 2005). The syndrome is characterised by global muscle weakness, severe learning difficulties, and an elevated serum T3.

The TRs, TRα or TRβ, are encoded by two distinct genes (*THRA* and *THRB* respectively). Both receptors have at least two splice variants: $TR\alpha_1$, $TR\alpha_2$ and $TR\beta_1$, $TR\beta_2$, although the $TR\alpha_2$ variant is functionally redundant (Yen 2001). Truncated variants of TRα (TRΔa1 & TRΔa2) (Chassande *et al* 1997), and sequence variants of TRβ (TRβ3 & TRβΔβ) are identified in rodents (Williams 2000), but their functional relevance is uncertain.

TRα1 and TRβ1 are widely expressed in thyroid hormone-sensitive cells. TRβ2 was originally thought to be expressed specifically in the hypothalamus and pituitary (where it has an inhibitory and negative feedback effect (Abel *et al* 1999)). However, it has since been recognised that TRβ2 plays an important role in sensorineural development of the cochlea and retinal cells (Jones *et al* 2007; Ng *et al* 2001 respectively).

1.5 TSH and its receptor

The TSH-TSHR interaction is a key event in thyroid biology as it precipitates the synthesis and release of thyroid hormones from the TG (Davies *et al* 2005). It also enables growth and development of the TG, particularly in postnatal life (Van Vliet 2003). The TSHR is present in many extra-thyroidal tissues including brain, testes, heart, kidney, lymphocytes, thymus, adipose tissue, bone and fibroblasts (reviewed in Davies *et al* 2002). However, its role in these tissues is uncertain and remains an area of research and debate.

The TSHR is the antigen against which thyroid autoantibodies develop. *Inactivating* antibodies block the TSHR and can cause SH or primary hypothyroidism (PH) whilst *activating* antibodies stimulate the TSHR (thyroid stimulating immunoglobulins; TSI) and can cause

Shyper or primary hyperthyroidism (i.e. Graves' Disease (GD)). The ratio of stimulating to blocking antibodies influences the clinical presentation (Ando *et al* 2005). The TSI-TSHR interaction may contribute to the pathogenesis of the non-thyroidal tissue manifestations of GD (Fatourechi 2005), these are discussed in section 1.7.1.

The TSHR is a G protein-coupled glycoprotein transmembrane receptor. The G-protein coupled receptor (GPCR) family consist of several thousand receptor types (Ji *et al* 1998, Fredrikssen & Schiöth 2005) ~850 are identified in humans (Fredriksson *et al* 2003, Bjarnadottir *et al* 2006). These receptors collectively respond to an array of chemical and biological signals which in humans include sensory perception (light, odours, taste), organic compounds (including neurotransmitters, hormones and chemokines), cations, toxins and drugs to name but a few. GPCRs are present in eukaryotes and fungi and also identified in some plants (Josefsson 1999).

The GPCRs have a common structural theme with an extracellular, a transmembrane and an intracellular portion (Hille 2009). The ectodomain begins with the amino terminal and may be glycosylated (the size and composition of the ectodomain can vary greatly between GPCRs (Tuteja 2009). The transmembrane portion (or serpentine portion) comprises a characteristic 7 transmembrane loop structure (with 3 extracellular and 3 intracellular portions) followed by an intracellular cytoplasmic section ending in the carboxyl terminus. The extracellular portion (+/- the ectodomain of the transmembrane loops) determine binding specificity (Barwell *et al* 2011). Whereas the serpentine section couples to the *G proteins* or *Guanine nucleotide binding proteins*.

G-proteins in GPCRs are heterotrimeric and composed of α, β and γ subunits. The α subunit is attached to a guanine residue which is *activated* when GDP (guanosine diphosphate) is exchanged for GTP (guanosine triphosphate) causing the α unit to dissociate from the βγ complex. The dissociated α subunit and (less commonly) the βγ complex initiate 2nd messenger signalling cascades (Hurowitz *et al* 2000), or directly stimulate physiological effectors (Kristiansen 2004). More than 20 Gα subunits are identified in mammals (Baltoumas *et al* 2013), all with diverse cellular effects. The β and γ subunits have several isoforms enabling hundreds of different heterotrimeric G protein combinations to be generated. The G-proteins are commonly described as *molecular switches* that switch on (stimulating

intracellular signalling pathways) when activated, and switch off when GTP is hydrolysed back to GDP. Ligand binding alters the GPCR structure, activating G-proteins and triggering intracellular signalling (Hamm 1998); thereby transducing signals from the extracellular to the intracellular environment.

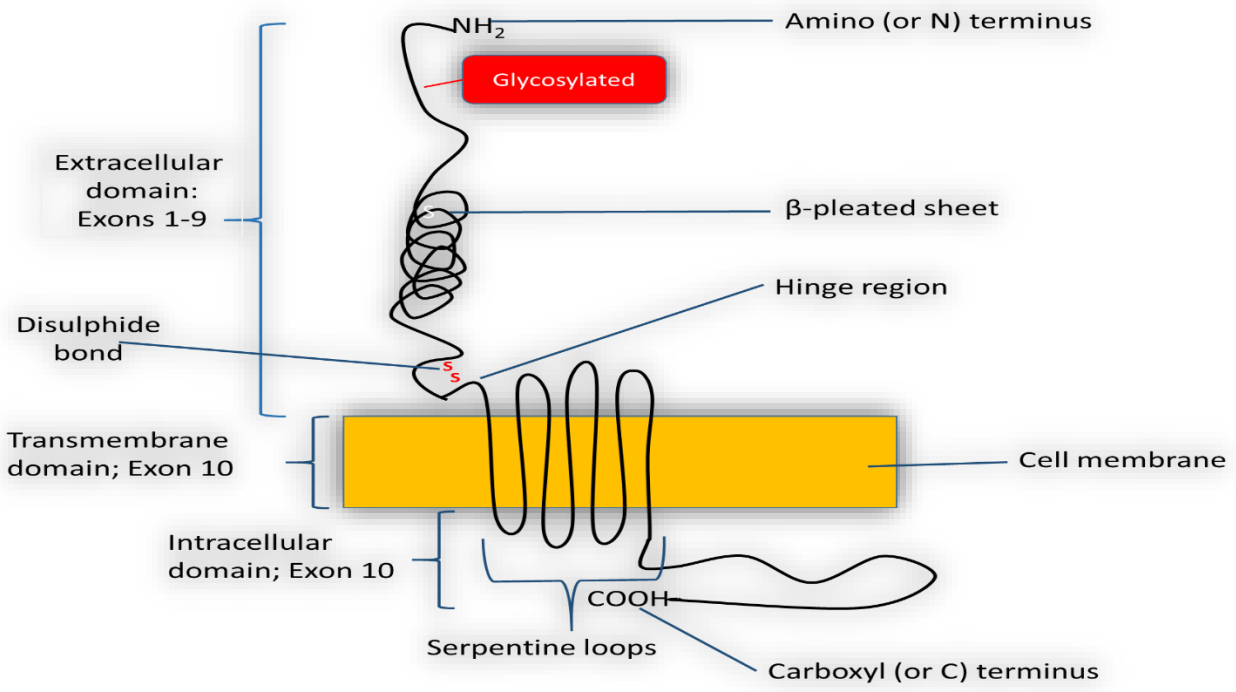
TSHR is a GPCR whose genetic code is spread across 10 exons, positioned on the long arm of chromosome 14 (genetic locus 14q31.1). It spans >190, 000 base pairs (Iosco & Rhoden 2009). The mature protein's molecular mass is 84.5kDa and consists of 764 amino acids (including a 21 amino acid signalling peptide). Of these amino acids 394 are extracellular, 83 intracellular, and those remaining occupy the transmembrane region (Nagayama *et al* 1989, Misrahi *et al* 1990). The first 9 exons code the extracellular domain (the main determinant of binding specificity (Braun *et al* 1991), whilst Exon 10 (that is more than twice the size of the other 9 exons combined (Iosco & Rhoden 2009) codes for the transmembrane and intracellular regions (Szkudlinski *et al* 2002).

The TSHR has a large ectodomain (relative to other GPCRs) with substantial sequence homology (35-45%) to the ectodomain of the gonadotrophin hormone receptors; follicle stimulating hormone (FSH), luteinising hormone (LH) and human chorionic gonadotrophin (hCG) receptors (Rapoport *et al* 1998). The gonadotrophin hormones and TSH are glycoprotein dimers with identical α subunits and distinct β subunits (Pierce & Parsons 1981). Therefore structural similarity in their receptors would be expected. The TSHR and the gonadotrophin receptor ectodomains are similar in that they are glycosylated and have 9 repeat leucine-rich regions that arrange themselves in β-pleated sheets (important to the quaternary structure of the receptor). Thus it might be anticipated that the gonadotrophin hormones, when produced to excess, might activate the TSHR. This is well described with hCG (particularly in the 1st trimester of twin and molar pregnancies when hCG precipitates a clinical and biochemical picture consistent with hyperthyroidism (Glinoer 1997, Hershman 1999)). However, this does not occur with FSH or LH.

Thyrostimulin is a relatively recently described glycoprotein heterodimer with specificity for TSHR (Nakabayashi *et al* 2002). It induces a response from TSHR that is similar in magnitude to TSH. Thyrostimulin is composed of α2 and β5 subunits. Localisation studies reveal its

presence in the anterior pituitary (Nakabayashi *et al* 2002) and ovary (Sun *et al* 2010) and a role as a paracrine activator, or modulator of TSH and/or TSHR function is suggested.

Figure 1.3: Illustration of the TSHR



The TSHR is depicted by a continuous black line extending from the N to the C-terminus. TSHR regions are labelled (in blue) and annotated as shown.

The TSHR is unique among GPCRs in that it is divided by post translational proteolysis into α (A) and β (B) subunits (Loosfelt *et al* 1992) with loss of a short C-peptide (Hamidi *et al* 2011). *In situ* they connect at the *hinge* region via disulphide bonds. The α-subunit consists of ectodomain (encoded by exons 1-8), whilst the β-subunit (encoded by exons 9 and 10) has a short extracellular component extending to the C-terminus (Van Durme *et al* 2006). Following TSHR cleavage, an excess of α-unit shedding leaves surplus β-subunits on the plasma membrane (Graves *et al* 1999) that can form dimers and oligomers (Latif *et al* 2001). β-subunit dimers are more prevalent on thyrocytes than extrathyroidal TSHR-expressing cells reflecting TSHR activity (Latif *et al* 2001). TSHR cleavage *is* associated with signal transduction although β-subunit dimers appear not to transduce signal (Ciullo *et al* 2003). Thus their functional significance is unknown.

The TSHR ectodomain and hinge region are described as a '*tethered inverse agonist'* to TSHR for the reason that the β-unit (in the absence of the α-unit) has constitutive activity (Vu *et al* 2009). Mutations in the hinge region can have similar effects (Zhang *et al* 2000, Vlaeminck-Guillem *et al* 2002). It therefore appears that the *hinge* and *extracellular domains* have a role in maintaining the receptor in a basal, unstimulated state. The TSHR is unusual amongst GPCRs in regard to its constitutive activation in the absence of ligand binding (Van Sande *et al* 1996).

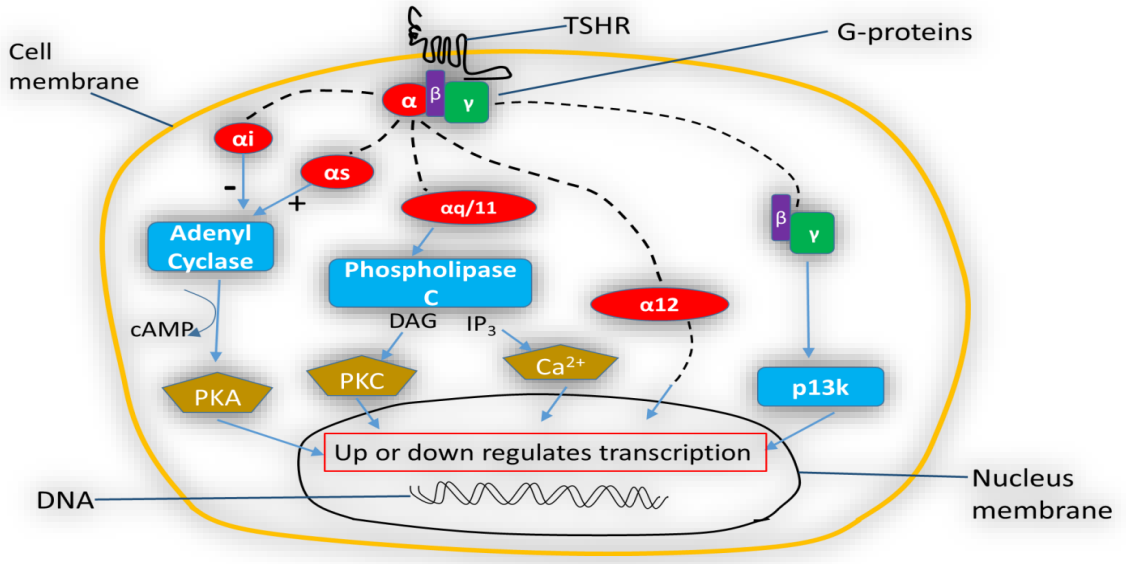
TSHR is considered a 'promiscuous receptor' as it can activate several distinct 2nd messenger signalling cascades (Allgeier *et al* 1994, Kleinau *et al* 2010). The best known is the Gαs-adenylyl cyclase-cAMP pathway (responsible for the upregulation of enzymes and proteins involved in thyroid hormone biosynthesis (Gilman & Rall 1968, Vassart & Dumont 1992)). Others include the Gαq/Gα11 pathway that stimulates the phospholipase C-inositol triphosphate (IP_3) /Ca^{2+} signalling cascades (Allgeier *et al* 1994); the Gαi (which has an inhibitory effect on adenylyl cyclase (Taussig *et al* 1993)) and the Gα12 (which influences the cytoskeleton amongst other effects (Majumdar *et al* 1999)). TSHR activates members of all 4 Gα protein subgroups (Laugwitz *et al* 1996), currently amounting to 11 different G-proteins (Holzapfel *et al* 2002).

Mice genetically engineered to lack Gαq/Gα11 develop hypothyroidism in infancy. Further investigation revealed that this pathway enabled upregulation of thyroglobulin pinocytosis and TPO-mediated iodide organification in response to rising TSH, and facilitated TG hypertrophy in response to a prolonged rise in TSH (Kero *et al* 2007). A subsequent case report confirmed an iodide organification defect in siblings homozygous for the *L653V* TSHR mutation, all with non-autoimmune SH. *In vitro* mutagenesis confirmed that the mutation caused a selective defect in IP_3 generation (Grasberger *et al* 2007). Thus demonstrating the importance of IP_3 signalling in TG biology. A ~100 fold higher TSH concentration is required to activate the IP_3 pathway, versus the cAMP pathway (Van Sande *et al* 1995). In recent years the importance of Gβγ signalling in TSHR cell biology has been recognised. This pathway modifies the P13K-PKB/ Akt pathway that is important in cell survival (Bell *et al* 2002) and adipogeneisis (Kumar *et al* 2011).

The methods used to decipher these intracellular sequencing cascades include observing the effects of: toxins (that selectively modify Gα-protein function (Hara *et al* 1999)), TSHR autoantibodies (agonists/ antagonists/ partial agonists/ neutrals) (Morshed *et al* 2009, Morshed *et al* 2010), and TSHR/ G-protein mutations (innate or induced) (Paschke & Ludgate 1997, Holzapfel *et al* 2002, Neumann *et al* 2005).

TSHR cell biology is more complex than I have suggested as the aforementioned cell signalling pathways cross-communicate with each other, as well as with other pathways (including STAT/JAK, NFkB/PKC and Akt/p13) (Morshed *et al* 2009). Therefore deciphering TSHR signalling cascades and unravelling their complex interactions and effects is challenging.

Figure 1.4: Principal TSHR intracellular signalling cascades



The dashed lines represent the signalling pathways, blue arrows and arrow heads indicate the target. Intermediary enzymes integral to the cascade are shown in blue and subsequent effectors/ enzymes are pentagons in brown. The diagram is labelled and annotated as shown.

Table 1.3: TSHR cAMP and phosphoinositide signalling pathways

Cell signalling component	TSHR signalling pathway	
	cAMP	Phosphoinositide
Activating G protein	As	αq/α11
Enzyme activated	Adenylate cyclase	Phospholipase C
2nd Messenger	cAMP	IP_3, (Ca^{2+}), DAG
Stimulated Protein Kinase	A	C
Target/ effectors	CREB (primarily)/ ERK	Akt/RAF/MEK/ERK

The abbreviations regarding 2nd messengers are as follows; Inositol 1,4,5-trisphosphate (IP_3), Diacylglycerol (DAG), Calcium (ca^{2+}).

1.6 Aetiology of Primary Hypothyroidism and Subclinical Hypothyroidism

Primary Hypothyroidism (PH) accounts for ~95% of hypothyroid cases (Baskin *et al* 2001) and is the most common endocrine disorder affecting ~2% of the population (1.9% female (♀) and 0.1 % male (♂) according to the UK Whickham survey (Tunbridge *et al* 1977)). It is ~10 times more common in women than men, and less common in African Americans than White Americans (NHANES study (Hollowell *et al* 2002)). Its incidence increases with advancing age (Vanderpump *et al* 1995).

SH has a prevalence of ~4-9% (Canaris *et al* 2000, Hollowell *et al* 2002) and is more prevalent in women than men (although sex ratios are more modest than PH affecting ~7.5% ♀ versus ~3% ♂ (Whickham Study (Tunbridge *et al* 1977)). Like PH, SH is more common with advancing age. Indeed the NHANES study reported a prevalence of 70% amongst subjects >80 years of age (defining SH as a TSH> 4.5mU/L).

Progression from SH to PH is variably quoted at ~3-18% per year (McDermont & Ridgeway 2001). A 20 year follow up of the Whickham study (included 1877 of the original 2779 subjects (defining SH as TSH>6 mU/L) reported that ~2.6% of antibody negative SH females progressed to PH annually, compared with ~4.3% per year if seropositive. In total, a third of the original SH group progressed to PH over a 20 year evaluation period. Interestingly, the risk of

progression for a man with SH and +ve antibodies was ~4x that of a woman (Vanderpump *et al* 2005). However, most subjects with SH remain in this category long-term suggesting that many have a *reset thyrostat* explaining their absence of progression to PH (Kabadi 1993).

The causes of SH are generally the same as PH. Worldwide iodine deficiency is the commonest cause (Patrick 2008). In iodine replete areas, traditionally thought to include the UK (although this notion has recently been questioned (Vanderpump *et al* 2011)) autoimmune thyroid disease (ATD) is the most common cause (Vanderpump 2011). Thereafter, iatrogenic causes predominate (eg. following thyroid surgery, radiotherapy, drug induced causes (including antithyroid medication)). Less common causes include thyroiditis (often recoverable) whose common aetiologies include postpartum-related, viral and drug-related. Infiltrative causes for SH and PH are rare but include Sarcoidosis, Amyloidosis, lymphoma and haemochromotosis-related causes (Bharaktiya *et al* 2012). Genetic causes for PH, although seemingly rare are an important group and one of the major causes for *congenital hypothyroidism* (CH). Genetic causes for SH are rarely explored.

ATD is believed to relate to genetic susceptibility (~70-80%) and environmental triggers (~20-30%), (Hansen *et al* 2006). Risk factors for developing autoimmune *hypothyroidism* include a family history (FH) of ATD (particularly >1 member), having positive TPO antibodies (risk increaing with the antibody titre (Strieder *et al* 2008, Brent 2010)) and possibly low birth weight (Phillips *et al* 2002). Environmental triggers include drugs (i.e; Amiodarone (Rabinowe *et al* 1986), Interferon-α (Carella *et al* 2004), antiretrovirals (Chen *et al* 2005), Campath-1H (Cossburn et al 2011) and radiation exposure (Eheman *et al* 2003). Other acquired causes include pregnancy (post-partum presentation typical (Muller *et al* 2001), iodine exposure (Foley 1992) and infections are implicated (Desailloud 2009, Tomer & Davies 1993).
Smoking is negatively associated with Hashimoto's thyroiditis and positively associated with GD (Krassas & Wiersinga 2006).

ATD is characterised by lymphocytic infiltration of the TG. In practice the diagnosis is based on clinical presentation and evidence for ATD (i.e. supportive biochemistry, immunology, histology or radiology (Weetman 2004)). Although most patients with ATD are TPO antibody +ve, a proportion are seronegative (Dayan & Daniels 1996). ATD evolves to subclinical disease that *may* progress to hypo- or hyperthyroidism. Autoimmunity occurs as a consequence of

loss of immune tolerance resulting in antigens from thyroid tissue being presented on antigen presenting cells. The antibody-antigen interaction stimulates an immune response (Chistiakov 2005) causing lymphocytic infiltration and inflammation of the TG (Devendra & Eisenbarth 2003). Thyroid antigens (with complementary autoantibodies) exist singly or multiply in affected individuals and include TSHR, TPO, Na/I symporter and thyroglobulin. TSHR antibodies in Hashimoto's hypothyroidism have a *blocking* effect, whilst in GD TSI are stimulatory.

CH describes hypothyroidism existing at birth, and typically diagnosed in the neonatal period. Primary CH is categorised as *dyshormonogenesis*, where the TG appears normal but its ability to produce thyroid hormone is compromised (due to thyroid biosynthetic defects). Whereas in thyroid *dysgenesis* thyroid hormone deficiency corresponds to an abnormally developed TG (hypoplasia, ectopy or athyreosis) (Park & Chaterjee 2005). Thyroid dyshormonogenesis and dysgenesis are distinguished radiologically and biochemically (with thyroglobulin assessment (Rastogi & LaFranchi 2010)). A significant delay in the diagnosis and treatment of severe CH (where thyroxine replacement therapy (TRT) is indicated) will result in irreversible learning difficulties. Routine screening for CH was introduced in the UK in 1981 (Department of Health and Social Security 1981) and CH is diagnosed in 1/3000 - 1/4000 infants (Gruters 1996). A CH female preponderance is recognised (LaFranchi *et al* 1979).

Dyshormonogenesis accounts for ~10-15% of cases of permanent primary CH and is believed to occur as a consequence of epigenetic and genetically determined defects in thyroid biosynthetic enzymes (Rastogi & LaFranchi 2010). This includes defects in TPO, Na/I symporter, thyroglobulin, Pendrin, DUOX2 and faults in the hydrogen peroxidase-generating system (Jordan *et al* 2003). Dyshormonogenesis tends to be autosomal recessive (AR), (Al Taji *et al* 2007).

Thyroid dysgenesis explains ~85% of cases of permanent primary CH (Rastogi and LaFranchi 2010). It is typically sporadic in presentation with ~2% of cases being familial (Castanet *et al* 2000). The cause remains elusive in most cases, reflecting limitations in knowledge of the developmental origins of the TG (Rastogi & LaFranchi 2010). Genetic causes include defects in the TSHR, Gsα, thyroid transcription factors (TTF) (including TTF-1, Pax-8, and FOXE1).

When the cause relates to Gsα or TTF defects a syndrome of abnormalities results. This reflects the non-organ specific developmental expression of these factors (Trueba *et al* 2005).

TSHR inactivating mutations can be carried in the homozygous or heterozygous state. Homozygous (or compound heterozygous) mutations are typically associated with CH (or SH with a markedly raised TSH; Alberti *et al* 2002) whereas heterozygous mutations (one affected and one unaffected allele) are typically associated with SH (and a mild to moderate TSH rise). The mode of inheritance is generally described as AR (de Roux *et al* 1996, Jordan *et al* 2003, Park *et al* 2004) and genotype/ phenotype correlations described as poor (even amongst subjects with the same mutation (Camilot *et al* 2005)).

The prevalence of heterozygous TSHR mutations (and variant alleles) in *non-autoimmune* SH has been explored infrequently. This was evaluated independently in two Italian paediatric cohorts where its prevalence varied from 11% (n=13/116; Camilot *et al* 2005) to 30% (n=11/38; Nicoletti *et al* 2009). This was evaluated in one adult cohort where sporadic cases and their SH relatives were screened. They reported a prevalence of 12% (n=5/42), and all cases were familial not sporadic (Tonacchera *et al* 2004). Jordan *et al* (2003) suggested that the prevalence of the TSHR nonsense mutation W546X (in its heterozygous state) affected ~1/180 in a Welsh population (without known thyroid dysfunction (affected 2/368).

It is likely that many cases of non-immune SH are genetically determined but rarely explored. These could relate to mutations (or possibly polymorphisms) affecting the thyroid biosynthetic and/or developmental enzymes; steps within the cell sequencing cascades, or relate to other factors influencing thyroid function. Studies suggest that TFTs in health are tightly regulated, and that *inheritance* plays a large part in determining these values. Estimates suggest that TSH values are ~65% hereditary and FTHs are ~40-50% hereditary (Panicker *et al* 2011).

Genome Wide Association Studies (GWAS) have identified *gene polymorphisms* that associate with TSH values. However their individual contributions to TSH on a population scale are small (<4%). Those receiving particular attention include FOXE1, the mineralocorticoid receptor (NR3C2), TSHR, and phosphodiesterase 8B (PDE8B). PDE8B catalyses the hydrolysis and inactivation of cAMP, thus increased PDE8B activity would be expected to precipitate a rise

in TSH. This was supported by association studies (Arnaud-Lopez *et al* 2008, Taylor *et al* 2011). In these studies the FOXE1 polymorphism was negatively associated with TSH (Medici *et al* 2011, Taylor *et al* 2015) as was D727E (the best studied of the TSHR polymorphisms (Panicker *et al* 2011)); whilst the NR3C2 polymorphism was positively associated with TSH (Rawal *et al* 2012, Taylor *et al* 2015).

1.7 Physical effects of thyroid dysfunction

The association between thyroid dysfunction and changes in body composition are well recognised. The English physician Sir William Gull in 1873 appears to have been the first to associate atrophy of the thyroid gland with the *hypothyroid* phenotype (Pearce 2006). The Swiss Surgeon Theodor Kocher was the first in 1888 to introduce TRT (by recommending consumption of sheep thyroid to thyroidectomised patients). Kocher attributed their hypothyroid symptoms to thyroidectomy (Kazi 2003). The Welshman Dr Caleb Parry in 1825, and the Irishman Dr Robert Graves in 1835 are recognised for their early descriptions of the *hyperthyroid* phenotype and for linking this to a thyroid goitre (hence the eponym *Graves' Disease* (GD), (Graves 1835)).

1.7.1 Hyperthyroidism

Hyperthyroidism (or thyrotoxicosis) results in a range of physical changes (reflecting this enhanced metabolic state) including weight loss (despite hyperphagia), muscle wasting +/- myopathies (Kim *et al* 2013) including cardiomyopathies; heat intolerance, loose stools (from enhanced gut transit), bone loss with increased fracture risk (Lee & Ananthakrishnan 2011), catecholamine hypersensitisation (Ginsberg *et al* 1981) with associated resting tachycardia and tendency to tachyarrhythmias, systolic hypertension (with risk of heart failure, atrial fibrillation (AF) and stroke (Osman *et al* 2002)), tremor, agitation and anxiety (Schraga 2012). Serological abnormalities may include hypercalcaemia, hyperglycaemia, deranged liver function tests (LFTs) particularly transaminases (Thompson *et al* 1978) and elevated alkaline phosphatase (ALP) (of bony or hepatic origin (Tibi *et al* 1989, Schraga 2012). Untreated hyperthyroidism curtails lifespan due to complications of the disease (particularly vascular events (Osman *et al* 2002)) or from systemic decompensation in the context of intercurrent illness (i.e. thyroid storm with a ~10-20% mortality despite treatment (Karger & Fuhrer 2008)).

Hyperthyroid effects in children include enhanced linear growth and bone maturity (Segni & Gorman 2001), which in neonates can cause craniosynostosis (Johnsonbaugh *et al* 1978). Hyperthyroidism in pregnancy increases complication risks, including pre-eclampsia, heart failure (Sheffield & Cunningham 2004), placental abruption, intra-uterine growth restriction, prematurity, stillbirth and neonatal mortality rates (Krude *et al* 1997).

Additional effects specific to GD include Graves' orbitoathy (GO) (affecting up to ~70% radiologically (Kahaly 2001), ~30% clinically (Tanda *et al* 2013) ~3-5% have severe disease (Wiersinga & Bartalena 2002)). GO signs and symptoms include dry, gritty eyes, periorbital oedema, exophthalmos +/- optic neuropathies and myopathies (with risk to vision and eye movement (Bahn 2010)). GD may be associated with *thyroid dermopathy* (localised myxoedema most commonly affecting pretibial skin (Lee & Ananthakrishnan 2011), affecting ~13% with severe GO (Fatourechi 2005). *Thyroid arcropachy* (disease of the extremities which become swollen, bulbous and severely clubbed) affects ~20% of those with thyroid dermopathy (Bahn 2010).

1.7.2 Hypothyroidism

The signs and symptoms generally depend on the degree of TG failure and age at onset. Symptoms may include weight gain, cold intolerance, brittle hair and nails, dependent oedema with thick, dry mottled, myxoedematous skin and tissues (including macroglossia), a gruff deepened voice, generalised muscle weakness, slowing of mental and physical processes (somnolence, cognition, mood, peristalsis) with *slowly relaxing reflexes* being a classic feature on neurological examination (Bharaktiya *et al* 2012). Menorrhagia and menstrual irregularities, erectile dysfunction in men, and sub-fertility in both sexes occur (Bharaktiya *et al* 2012). Physical signs may include goitre, myxoedematous tissue and facies, hypertension (particularly diastolic with narrowed pulse pressure (Danzi & Klein 2003)), bradycardia and bradyarrhythmias, heart failure (Fazio *et al* 2004) and obstructive sleep apnoea. In recent years an increased risk of bone fracture (which may not correct with treatment of hypothyroidism) is reported (Vestergaard & Mosekilde 2002, Vestergaard *et al* 2005).

Hypothyroid abnormalities revealed from blood tests include hyponatraemia, hyperprolactinaemia, elevated creatine kinase, susceptibility to hypoglycaemia, normocytic anaemia (Bharaktiya *et al* 2012), hyperlipidaemia (Walton *et al* 1965) and deranged LFTs (Huang and Liaw 1995). Profound untreated hypothyroidism leads to premature death either from complications of the disease (i.e. vascular events relating to accelerated atherosclerosis), or through a compromised ability to deal with the demands of often minor stresses, as seen in myxoedema coma (Mathew *et al* 2011).

Hypothyroidism in children has additional effects reflecting its impact on growth and development. This includes compromised growth, immature teeth and bones and delayed epiphyseal closure (with risk of *slipped epiphysis* (Foley 2004)), muscle pseudohypertrophy (Klein *et al* 1981, Shaw & Shaw 2012) (rare in adults) and sexual immaturity (although reversible precocious puberty is described (Foley 2004)). Intellectual development is compromised with risk of permanent deficits when treatment is not initiated in a timely manner (particularly <3 years of age (Foley 2004)). Adverse effects on mental functioning (thought processing, concentration and memory) will compromise a child's ability to learn, although this is usually reversible with TRT.

Hypothyroid infants can display the following additional signs: open cranial fontanelles, feeding and breathing difficulties, jaundice, hypotonia and umbilical hernia (Rastogi & LaFranchi 2010). Historically individuals living with the consequences of untreated infant hypothyroidism were referred to as "Cretins", in reference to their physical and mental disabilities (Bulugahapitiya 1972).

Hypothyroidism in pregnancy carries additional risks for the mother and foetus (relating to gestational stage at onset) including an increased risk of miscarriage, stillbirth, pre-eclampsia, placental abruption (Mooney *et al* 1998), perinatal mortality (Van den Boogaard *et al* 2011) and compromised IQ in offspring (Haddow *et al* 1999, Smallridge & Ladenson 2001).

Hyperthyroidism and hypothyroidism are therefore associated with significant harm untreated and correction is always recommended. In contrast, the benefit of correcting subclinical thyroid diseases (ScTD) is less clear, accounting for considerable heterogeneity in their clinical management (McDermott *et al* 2003).

1.7.3 Subclinical Hyperthyroidism

The recommendations are generally to treat *persistent* Shyper in older adults (>65 years; Cooper 2007, Bahn *et al* 2011), subjects with suboptimal bone mineral density (BMD), particularly postmenopausal women (Faber *et al* 1998, Mudde *et al* 1994, Bahn *et al* 2011); subjects experiencing tachyarrhythmias (Forfar *et al* 1981) or those with co-morbidities who would be compromised by hyperthyroidism or tachyarrhythmias (Haentjens *et al* 2008). The risk of progression to hyperthyroidism may also influence this decision (a higher risk with an unmeasurable TSH (versus a sub-normal TSH (Diez & Iglesias 2009)) and with GD versus nodular thyroid diseases (NTD) (Rosario 2008).

These recommendations are based on supportive data and expert opinion (Bahn *et al* 2011, Yang *et al* 2012). However, evidence to show that these risks abate with treatment are generally lacking (Surks *et al* 2004, Blum *et al* 2015).

1.7.4 Subclinical Hypothyroidism

The international guidelines are generally unified in their recommendation to treat *persistent* SH when the TSH remains >10mmol/L (i.e. British Thyroid Association, American Association of Endocrinologists, and The Endocrine Society (Surks *et al* 2004, Garber *et al* 2012)), particularly in subjects <65 years of age (Pearce *et al* 2013). At this threshold the evidence for a benefit from treating SH in regard to improvements in non-HDL cholesterol (Meier *et al* 2001, Pearce *et al* 2013), cardiovascular risk parameters (Rodondi *et al* 2010) and avoidance of future hypothyroidism (~10% progress to hypothyroidism within 4 years (Somwaru *et al* 2012)) are considered satisfactory. However, below this threshold much uncertainty remains.

In the case of mild SH (TSH <10mU/L) it is generally recommended that subjects should be *considered for treatmen*t on an individual basis (Gharib *et al* 2005). This group emphasise that a *lack* of good quality studies to support a treatment benefit (i.e. mood/ cognition (Jorde *et al* 2006), hypertension (Surks *et al* 2004, Fatourechi 2009), dyslipidaemiaa (Pearce 2012)) are not a reason to refute it (villar *et al* 2007). They particularly advocate treating subjects with comorbidities that are known to improve with treatment of hypothyroidism (including hypertension, dyslipidaemia and vascular diseases), and those describing hypothyroid symptoms that improve following a trial of TRT (Pearce *et al* 2013).

Pregnant women with SH are considered separately and correction of mild SH (to pregnancy-specific targets) are recommended (Beastall *et al* 2006, Abalovich *et al* 2007). The data to support this practice, although adequate, are by no means consistent across studies (Cleary-Goldman *et al* 2008, Reid *et al* 2010). There is currently insufficient evidence to advocate screening women seeking pregnancy (or in early pregnancy) for thyroid dysfunction (Lazarus *et al* 2012). However studies that may challenge this practice are in progress (including the Cardiff Antenatal Thyroid screening study (CATS) which is re-testing childhood cognitive function at age 9).

The extreme elderly (>85 years) should also be considered separately in regard to TRT decisions in mild SH. It is suggested that these subjects are less likely to progress to hypothyroidism, are usually asymptomatic, and a higher TSH and lower T4 show favourable mortality associations (Meneilly 2005). Therefore routine treatment is not recommended (Pearce *et al* 2013). A study by Razvi *et al* (2008) evaluated cardiovascular outcomes in a meta-analysis of SH subjects and reported adverse associations for those <65 years of age but not above 65 years. These observations may reflect the rise in TSH with advancing age (Bremner *et al* 2012), indicating that it may be physiological in many aging subjects.

1.8 Hypothesis building

SH is a heterogeneous and prevalent condition where its natural history and optimum management may be aetiology specific. The controversies and uncertainties surrounding this subject area highlight the need for greater research. In the relatively recent past there have been an influx of studies (predominantly *in vitro* and animal studies) indicating that TSHR expression in extra-thyroidal locations might influence cellular processes locally.

This new perspective questions traditional thoughts that attribute all of the physical changes occurring in thyroid disorders to aberrant FTHs. Instead, in *primary* thyroid diseases the more substantially and reciprocally altered TSH may be playing a part.

Extra-thyroidal TSHR may have a functional or developmental role in these locations. To explore this possibility further research has been conducted that aims to modulate TSHR activation (in cells, tissues or organisms) whilst seeking to minimise change in FTHs.

My personal interest relates to potential TSHR related effects that may be relevant to body composition components (namely bone, fat (or adipose tissue) and muscle). Research that relates to these subject areas will now be discussed.

1.9 Bone

Hyperthyroidism (Lee & Ananthakrishnan 2011) and to a lesser extent Shyper (Faber *et al* 1998, Mudde *et al* 1994) are associated with accelerated bone turnover (BT) and bone loss; whereas hypothyroidism is associated with delayed bone maturity, increased fracture risk, and compromised bone healing (Murray 1900, Vestergaard P and Mosekilde 2002). It is likely that SH also has effects on bone although this is less clear.

An important bone disease from a socioeconomic perspective, in developed countries is osteoporosis. Osteoporosis describes thin fragile bones with a propensity to fracture, however their mineral content is normal (Glaser & Kaplan 1997). Osteoporotic fractures are common in older people affecting ~50% of women and ~20% of men over fifty (Kanis & Johnell 2005). Osteoporotic fractures impose significant morbidity with ~50% losing independence (Eddy *et al* 1998) and ~25% dying within a year of hip fracture (Cooper 1999). In the UK the cost of osteoporotic hip fracture was estimated above ~2 billion pounds per year in 2007 (British Orthopaedic Association 2007). Thus associations between thyroid disorders and bone health, particularly those that may relate to SH (the most common thyroid condition) would be important from a public health and economic perspective.

1.9.1 Background on bone cell biology

Bone and bone cells function in a coordinated manner and adapt to mechanical and metabolic demands. Bone is resorbed and renewed in a regulated fashion, *coupling* being the term to describe this process (Parfitt 1982). Bone remodelling is considered in phases; “activation”

when osteoclasts (and precursors) are drawn to the site, “resorption” mediated by osteoclasts, “reversal” accumulation of osteoblasts and precursors, “formation” mediated by osteoblasts, and ‘quiescence’ the time lag before the next cycle starts (Parfitt 1984). In adults a remodelling cycle lasts ~150-200 days (Wojcicka *et al* 2013) and is accelerated ~30% in thyrotoxicosis (Eriksen *et al* 1985). Thyrotoxicosis *uncouples* BT with greater effect on resorption than restoration resulting in a ~10% reduction in bone volume per cycle (Mosekilde *et al* 1990). If remodelling remains uncoupled, metabolic bone diseases ensue. The cells that mediate bone remodelling and restoration are the chondrocytes, osteoblasts, osteoclasts and osteocytes.

Chondrocytes are cartilage forming cells that enable longitudinal growth (Hinchliffe & Johnson 1983) until the cartilaginous growth plates (epiphysis) close on attainment of final height (Haas 1917). The mammalian skeleton is ossified from a cartilaginous framework (Robinson & Cameron 1956). Many factors control this process including the transforming growth factor (TGF-β) superfamily (including bone morphogenic proteins (BMPs)) and the vascular endothelial growth factors (VEGF), (Yang *et al* 2012). Epiphyseal chondrocytes go through phases of maturation; proliferation, differentiation (to mature hypertrophic chondrocytes that synthesis cartilage) and apoptosis. Apoptosis is followed by cell infiltration enabling ossification to occur (Rabier *et al* 2006). Cartilage persists at the ends of adjoining bones with a role in dissipating force and protecting the joint and bone surface (Eckstein *et al* 2006).

Osteoblasts are responsible for bone formation (Millett *et al* 1995) and are derived from mesenchymal stem cells (MSCs) originating in bone marrow. MSCs can differentiate into distinct terminal cells (Friedenstein *et al* 1974; Millett *et al* 1995) including myocytes, chondrocytes, adipocytes and osteoblasts (Y Zhang *et al* 2012). Osteoblasts synthesise organic bone matrix (osteoid) and mineralise osteoid to mature bone (Millet *et al* 1995). Osteoblasts express the TNF-related protein RANKL (receptor activator of nuclear factor κβ ligand (Anderson *et al* 1997)) on their surface (Yasuda *et al* 1998). RANKL interacts with osteoclast RANK receptors (with stimulatory effect) whilst its soluble variant encourages osteoclast differentiation. M-CSF (Macrophage Colony-Stimulating Factor) is another osteoblast synthesised cytokine encouraging osteoclast differentiation (Tanaka *et al* 1993). Osteoprotegerin is a tumour necrosis factor (TNF) related protein (Simonet *et al* 1997, Yasuda

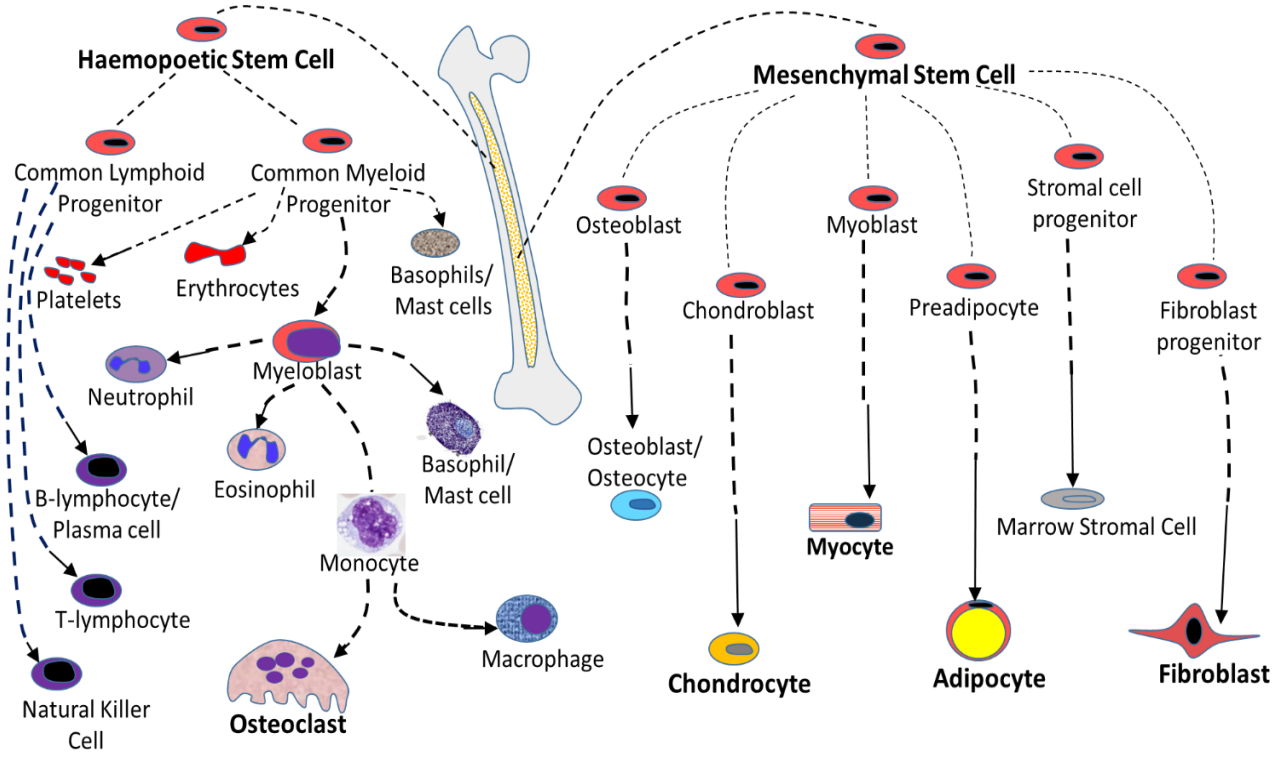
et al 1998) secreted by osteoblasts that interacts with RANKL (on its own surface), sterically hindering association with RANK (on osteoclasts) thereby controlling bone resorption. The transcription factors Runx2 and osterix have a vital role in osteoblast differentiation (Sinha & Zhou 2013).

Osteoclasts are multi-nucleated cells derived from the macrophage-monocyte lineage of heamopoetic stem cells (HSCs) in bone marrow (Boyle *et al* 2003). Osteoclasts are responsible for dissolving and resorbing bone (Chambers & Hall 1991) with release of calcium and phosphate to the extracellular compartment (Silver *et al* 1998).

Osteocytes are osteoblasts trapped in bone, ~10-20% of osteoblasts become osteocytes (Noble 2008). Osteocytes produce proteins that influence bone behaviour locally including BT and biomineralisation (Bonewald 2011). This is accomplished via a network of appendages radiating from the osteocyte. Osteocytes produce many cytokines including TGF-β that suppresses bone resorption and encourages osteocyte formation (Mundy 1991), and sclerostin that suppresses osteoblast activity (Atkins *et al* 2011). Sclerostin mediates its effects by interacting with the osteoblast's Wnt co-receptors (LRP5 and LRP6), thereby moderating Wnt signalling (Silverman 2010).

Wnt describes a family of secreted signalling proteins, highly conserved through evolution. They mediate effects in an autocrine or paracrine fashion and bind Wnt cell surface receptors (Krishnan *et al* 2006). Wnt signalling occurs via the *non-canonical* and *canonical* pathways to moderate transcription of Wnt responsive genes (MacDonald *et al* 2009). Wnt signalling is important in the growth, differentiation and maintenance of tissues, including bone (Krishnan *et al* 2006).

Figure 1.5: Flow diagram illustrating the differentiation pathways and terminal cell types originating in bone marrow



In figure 1.5 the terminal cells are indicated by arrow heads, dashed lines indicate the differentiation pathways followed (which commonly involve several differentiation steps) originating from bone marrow.

Wnt signalling has osteogenic effects. This includes encouraging differentiation of MSCs to preosteoblasts (versus preadipocytes; Song *et al* 2012, Hamidouche *et al* 2008), encouraging osteoblastogenesis (Krishnan *et al* 2006), hindering osteocyte and osteoblast apoptosis (Bodine *et al* 2004) and suppressing osteoclast activity (Spencer *et al* 2006). Inhibitors of the canonical pathway include secreted frizzled related proteins (Frz) that interfere with frizzled receptors i.e. dickkopf (Kawano & Kypta 2003) and sclerostin (Silverman 2010). The importance of Wnt signalling is evident when these pathways are disrupted. Loss of the LRP5 co-receptor causes profound early onset osteoporosis in the osteoporosis-pseudoglioma syndrome (Gong *et al* 2001), whereas mutations in LRP5 that reduce dickkopf binding cause a high bone mass phenotype (Ai *et al* 2005, Boyden *et al* 2002). Abnormal signalling in the non-canonical pathway (i.e. Wnt 5b) are a cause of Robinow syndrome (features include craniofacial dysostosis, shortened long limbs and genital defects (Person *et al 2010*). Thus the *canonical* and *non-canonical* Wnt pathways are important in bone biology.

1.9.2 Thyroid hormones and bone biology

FTHs have profound effects on bone. T3 has a stimulatory effect on osteoblasts primarily mediated by TRα1 (Bassett & Williams 2009). TRα1 is the most prevalent of the osteoblast TRs, being ≥10 times as prevalent as TRβ (Xing *et al* 2012, O'Shea *et al* 2003) although other TRα and β splice variants are expressed (Milne *et al* 1999). It is suggested that TRβ may mediate the immediate osteoblast response to a change in FTHs whilst TRα1 mediates the longer term response (Monfoulet *et al* 2011).

Osteoclasts are stimulated by T3. This appears to be through moderating the osteoblast RANKL/ osteoprotegerin ratio (as T3-osteoclast experiments in the absence of osteoblasts generally fail to show an effect). TRs are probably expressed on osteoclasts (based on the extraction of TR mRNA from osteoclastoma cells (Allain *et al* 1996) and osteoclasts (Abu *et al* 2000)).

Chondrocytes are influenced directly and indirectly by FTHs (FTHs moderate the expression of Indian Hedgehog protein/PTH-related hormone and receptor expression for GH, IGF-1, and fibroblast growth factor (Bassett & Williams 2009)). Hypothyroid epiphyses display disorganised chondrocyte columns and altered cartilaginous matrix, whilst this is essentially normal in hyperthyroidism (Stevens *et al* 2000, Freitas *et al* 2005). FTHs accelerate hypertrophic chondrocyte differentiation but suppress chondrocyte proliferation (Robson *et al* 2000). TRα1, TRα2, and TRβ1 are expressed on chondrocytes (Ballock *et al* 1999); with the exception of terminal hypertrophic chondrocytes (Robson et al 2000). The effect on epiphyses of knocking out TRs in mice (Bassett & Williams 2009) suggests TRα as the predominant receptor (particularly in long limbs). However, TRβ predominates in neonatal mouse rib (Rabier *et al* 2006) and the TRβ agonist GC-1 ameliorates the skeletal effects of hypothyroidism (Freitas *et a*l 2005).

Deiodinase activities alter in bone cells in response to altered FTH levels. *In vitro* studies confirm the presence of functional D3 in osteoblasts, osteoclasts and chondrocytes (Williams *et al* 2008). D2 is the only activating deiodinase detected in bone, with expression restricted to mature osteoblasts (Williams *et al* 2008, Morimura *et al* 2005). *DI02* knockout mice have

increased bone fragility attributed to prolonged bone remodelling precipitating pathological mineralisation (Bassett *et al* 2010).

DI02 expression is reported to increase in osteoblasts and human osteoblast-like osteosarcoma in vitro in response to TSH (forskolin or dibutyryl cAMP (Morimura *et al* 2005)). Upregulated DIO2 expression in response to TSH is also reported in other tissues including skeletal muscle (Hosoi *et al* 1999), rat brown fat (BAT) (Murakami *et al* 2001a) and thyroid tissue (Murakami *et al* 2001b).

1.10 TSHR and bone

The presence of TSHR on osteoblasts was first suggested by Inoue *et al* (1998). Experiments on a rat osteosarcoma cell line (UMR106 cells) confirmed; TSHR mRNA expression (by Northern blotting); a cAMP response to synthetic TSH (or TSI), and radio labelled TSH binding. They also demonstrated a TSH-induced cAMP response from human osteosarcoma cells and mouse osteoblasts. This group proposed a link between TSHR expression in bone and accelerated bone loss in GD.

Tsai *et al* (2004) compared TSHR expression in osteoblast-like cells versus TSHR transfected CHO (Chinese Hamster Ovary) cells. An osteoblast response required a 10-fold higher TSH concentration than transfected cells (correlating with TSH binding). TSHR mRNA was demonstrated in human bone samples using reverse transcription polymerase chain reaction (RT-PCR), but not with the more specific RNase protection technique. This group suggested that osteoblast TSHR expression was probably insufficient to contribute to thyroid bone disease.

Abe *et al* (2003) were the first to provide *in vivo* data indicating that TSHR has a functional role in bone. This group examined BMD, bone histology and bone cell behaviour in TSHR null mice (-/-), heterozygotes (-/+) and wild types (WT), (+/+). Null mice had CH with several thousand fold excess of TSH. They were small, osteoporotic and died at 10 weeks. Heterozygotes were macroscopically normal with TFTs in the euthyroid range (T3s similar, mean T4s ~↑70%, mean TSHs ~↑30% although differences were not statistically significant). Heterozygotes were macroscopically normal but had low BMD. The null mice were re-

examined following TRT at weaning. Although their growth, weight and TFTs at 6 weeks were similar to WT (T4s and TSHs were similar, T3 ~↑15% although not statistically dissimilar) BMD remained low. Histological assessment of the null mice on TRT revealed woven rather than lamellar bone (indicative of high BT), focal osteosclerosis and a 50% increase in mineralisation sites.

Evaluation of bone marrow cultures in the null and heterozygous mice revealed a doubling of osteoclastogenesis (believed to be mediated by macrophage TNFα) and increased osteoblast differentiation (probably Wnt and VEGF mediated). Experiments assessing WT cell responses to TSH indicated reduced osteoclast activity and osteoblast activity (reflected by protein expression profiles), whereas a response was absent in the TSHR null cells. This group concluded that TSHR was functional in bone and described TSHR as a "negative regulator of skeletal remodelling" (Abe *et al* 2003). The observations of this group has since been supported by a similar study (Zhang *et al* 2014).

Interestingly, a CH child in our unit (homozygous for the inactivating W546X TSHR mutation (TSHR-M)) was diagnosed with a benign osteoblastoma (unpublished observation JW Gregory & M Ludgate in de Lloyd *et al* 2010). The pathogenesis of this condition would fit with the suggestion that diminished TSHR function in bone upregulates osteoblast activity (Abe *et al* 2003).

Sampath *et al*'s work supports the notion that enhanced TSHR signalling in bone has osteogenic effects. This group evaluated aged, ovariectomised rats who were administered intermittent intraperitoneal TSH for 8 weeks (versus non-ovariectomised controls). This was initiated early post ovariectomy (n=80), or 7 months later (n=152). Exogenous TSH was found to lessen ovariectomy-induced bone loss, and enhance bone accrual and strength in the ovariectomised groups. Interestingly this treatment did not affect FTHs (Sampath *et al* 2007).

Analysis of the ovarectomised rodent's bone marrow indicated a suppressive effect on osteoclast differentiation, and an enhanced effect on osteoblast activity (not seen in non-ovariectomised controls). The group concluded that exogenous TSH has anabolic and anti-resorptive effects on hypogonadal rodent bone (Sampath *et al* 2007). A subsequent paper by this group advocated exogenous intermittent TSH as a potential future therapy for post-menopausal osteoporosis (Sun *et al* 2008).

The mechanism by which TSH infusions exert anabolic effects on rodent skeleton have been explored. A favourable osteprotogerin-RANKL ratio probably explains enhanced differentiation of MSCs to osteoblasts; a dose-dependent increase in non-canonical Wnt components during osteoblast differentiation was observed (Frz and Wnt5a), and is expected to increase osteoprotegerin locally. The effect on MSC differentiation is believed to be PKC pathway mediated (given that upregulated PKC phosphorylation was observed (on Western blots versus no change in PKA); abrogated by the PKC inhibitor rotterlin (Baliram *et al* 2011)).

Mazziotti *et al* (2005) evaluated the effect of exogenous recombinant TSH (rhTSH) on thyroidectomised subjects with Shyper (on TRT) with history of thyroid cancer (n=66; 38 pre-menopausal, 28 post-menopausal). Bone parameters were compared against age-matched, healthy controls (with no thyroid disorders) with comparable FTHs. Baseline assessments showed elevated osteoprotegerin and BTMs, and lower BMDs (assessed at finger phalange) in Shyper subjects relative to controls. The postmenopausal Shyper subjects had lower BMD and elevated bone-specific ALP. RhTSH caused TSH >100mU/L at day 2 post administration, without a change in FTHs. At day 2 an acute inhibition of bone resorption markers in the postmenopausal but not the pre-menopausal group was seen, associated with a rise in ALP. BTMs normalised at day 7 but ALP remained raised. Osteoprotegerin was unaffected. This group concluded that TSH influences bone physiology in humans and its effects are more apparent in post-menopausal women who are susceptible to Shyper induced bone loss.

1.10.1 Human studies exploring TSH-bone relationships

These data are less robust than the animal data and in the main relate to observational association studies. It is clear that there is much discrepancy between studies and the effect of TSH on bone (as distinct from the FTHs) remains uncertain. I have summarised several bone-related studies that assessed TSH-bone associations:-

- Table 1.4 reports adverse associations between Shyper and bone parameters.
- Table 1.5 reports adverse associations between low-normal TSHs (in euthyroid subjects or those without known thyroid dysfunction) and bone parameters.
- Table 1.6 reports positive associations between TSH and bone parameters.

Table 1.4: Studies reporting TSH-bone associations in subclinical hyperthyroidism

Paper	Summary of study	Key observation
Lehmke *et al* 1992	Evaluated BMD in Shyper subjects related to TRT and a past history of thyroid cancer (n=55; 9♂, 25 pre-menopausal♀, 21 post-menopausal♀). BMDs were compared with a healthy reference population.	BMD deficits were reported in the appendicular skeleton of post-menopausal♀ only.
Mudde *et al* 1994	Evaluated 20 postmenopausal♀ with Shyper related to NTD, 8 were treated with Carbimazole (to normalise TSH). Forearm BMD and BTMs were assessed at baseline and over 2 years.	BMD was higher in the distal forearms of the treated subjects. There were no differences in BTMs.
Guo *et al* 1997	Evaluated BMD and BTMs in postmenopausal♀ on TRT (n=64) over a 2 period (23 euthyroid, 41 Shyper). Those with Shyper had their TRT doses amended to normalise TSH.	Beneficial changes in BMD and BTM were seen in those in whom suppressed TSHs were normalised.
Bauer *et al* 2001	Evaluated fracture incidence in postmenopausal♀ >65 years (n=686). TSH was assessed in subjects with new fractures (148 hip, 149 spine) versus 398 randomly selected controls. FTHs were not evaluated.	TSH <0.01mU/L was associated with a 3 fold increase in hip fracture and 4 fold increase in lumbar fractures.
Kisakol *et al* 2003	Evaluated BTMs and calcium excretion in patients with SH (n=20), Shyper (n=13, all related to GD) and euthyroid controls (n=10).	BTMs and calcium excretion were elevated in subjects with Shyper only.
Svare *et al* 2009	HUNT2 data on 5778♀ >40 years of age. All had TFTs and BMD assessed at the distal forearm.	A low TSH (<0.5mU/L) associated with lower BMD. A history of hyperthyroidism associated with a higher incidence of osteoporosis.
Blum *et al* 2015	Meta-analysis evaluating fracture risk and subclinical thyroid conditions. Collective n= 70,298 (Prevalence: Shyper ~3%, SH~6%).	Associations between Shyper and fractures reported (HR 1.28, C.I; 1.06-1.5), but not for SH.

In tables 1.4-1.6 the abbreviations and symbols are as follows: ♀; Female, ♂; Male. BTMs; bone turnover markers, BMD; bone mineral density, TRT; thyroxine replacement therapy. Shyper; subclinical hyperthyroidism. SH; subclinical hypothyroidism. HR; hazard ratio. C.I; confidence interval, FTHs; free thyroid hormones. TFTs; thyroid function tests.

Table 1.5: Studies reporting adverse associations between low-normal TSH and bone parameters (in subjects euthyroid, or without known thyroid dysfunction)

Paper	Summary of study	Key observation
Bertoli *et al* 2002	Bone mineral content and BMD assessed in 32 pre-menopausal♀ alongside anthropometric data and TFTs.	A positive association between femoral BMD and TSH reported (but no independent associations at the spine).
Kim *et al* 2006	Evaluated BMD and TFTs in 959 healthy, post-menopausal Korean♀. They compared those with low-normal (0.5-1.1) versus high-normal TSHs (2.8-5mU/L).	Association between low-normal TSHs and; incidence of osteoporosis (95% C.I; 1.2-4) and lower BMD (at lumbar spine and femoral neck).
Grimnes *et al* 2008	Evaluated BMD (hip and forearm) and TFTs in healthy older adults (993♀ and 968♂).	Reported a negative association between BMD at distal forearm and sub-normal TSH (n=46); a positive association between BMD at femoral neck and TSH >97.5th% in ♀s (n=25). No associations between normal range TSHs and BMD seen.
Morris 2007	American NHANES data (included BMD and TFTs) on 581 postmenopausal♀.	A higher rate of osteoporosis (x 5) and osteopenia (x 3) was apparent amongst those with low-normal TSHs (0.39-1.8) versus higher-normal TSHs (1.8-4.5mIU/L). TSH correlated positively with BMD.
Murphy *et al* 2010	Evaluated BMD and TFTs in 1278 healthy post-menopausal♀.	Non-vertebral fracture incidence correlated negatively with TSH (minus 35%; P-value 0.028) and positively with T4. Hip BMD correlated negatively with T4. However, the independent influence of TSH could not be determined.
Mazziotti *et al* 2010	Evaluated 80 postmenopausal ♀s newly referred to an osteoporosis clinic.	Association between low-normal TSH (0.66-1.07 mU/L) and vertebral fracture seen (versus TSH 1.13-3.2mU/L (p-value 0.004)). This association persisted despite correction for T4.
Chin *et al* 2013	Chinese/ Malaysian cross-sectional study evaluating TFTs and calcaneal BMD in adult men with no known thyroid disorders.	Positive correlations between indicators of heel BMD and TSH were identified (R +0.08, p-value 0.04, n=681).

Table 1.6: Studies reporting an adverse association between subclinical hypothyroidism and bone parameters.

Paper	Summary of study	Key observation
Lee *et al* 2006	BMD and TFTs were evaluated in ♀subjects (n=413) without hyper or hypothyroidism.	BMD was significantly lower (at femoral neck not lumbar spine) in those with Shyper or SH versus euthyroid subjects.
Nagata *et al* 2007	TFTs and calcaneal BMD evaluated in SH postmenopausal♀ (n=22) versus euthyroid controls.	A negative association between calcaneal BMD indicators and TSH was reported and a lower bone score observed in SH subjects than controls.
Lee *et al* 2010	Evaluated 3567 US subjects >65 years prospectively (over 13 years) excluding those with hyper and hypothyroidism.	Fracture incidence was higher for males with endogenous Shyper (HR 5; 95% C.I; 1.1-21) and endogenous SH (HR 2.5 (95% C.I; 1.3-4.7) than euthyroid controls. This association was not seen in females.
Polovina *et al* 2013	FRAX score (fracture risk predictor) and BTMs were compared in post-menopausal♀ with SH (n=82) versus euthyroid controls.	FRAX score was higher in subjects with SH and particularly those with thyroid autoimmunity. BTMs were no different.
Liang *et al* 2014	Evaluated BMD, TFTs and bone profile in SH subjects (n=122) and controls.	SH was associated with lower BMD.

The Abe *et al* paper (2003) that linked TSHR activation in bone to bone health led researchers to evaluate bone parameters in subjects bearing the TSHR D727E polymorphism. This polymorphism is associated with *lower* TSH values (Peeters *et al* 2003) suggesting increased TSHR sensitivity. Consistent with this hypothesis Gabriel *et al* (1999) reported an exaggerated cAMP response to TSH versus WT, however this finding was not reproduced by other groups (Nogueira *et al* 1999).

Van Der Deure *et al* (2008) evaluated associations between BMD (at lumbar spine and hip) with the D727E polymorphism in a prospective cohort of 4801 subjects >55years of age without known thyroid diseases. TFTs were assessed in 1089 subjects. The D727E allele

frequency was ~6% (~11% heterozygous and 0.2% homozygous). Femoral neck BMD was reported to be ~2% higher in D727E carriers (0.88 ± 0.01 versus 0·86 ± 0·01, p-value 0.03), an association that strengthened with correction for TSH. In this study TSH showed a weak positive association with BMD, and T4 a negative BMD association (Van der Deure *et al* 2008).

A Chinese study by Liu *et al* (2012) evaluated the prevalence of D727E in a cohort of osteoporotic male subjects (n=150) versus healthy non-osteoporotic controls. Its prevalence was ~9% in osteoporotics and ~5% in controls (p-value <0.05). They reported lower BMD indicators at the calcaneum in the D727E carriers. Their conclusion opposed that of the previous group associating this polymorphism with lower BMD values.

1.10.2 TSHR-bone data not indicating an independent association with TSH

A number of academics remain sceptical or opposed to the notion that TSH-TSHR activation in bone has physiological effects that are independent of FTHs. One of the principal counter arguments relates to the reciprocal relationship between TSH and T4 (in subjects with an intact HPT axis) making it difficult to delineate the independent influence, *in vivo*, of the respective hormones. Therefore, when interpreting subclinical thyroid data, although the FTHs are considered to be normal relative to the population range, in Shyper they are relatively high (relative to the individuals preferred set point) and the inverse true of SH. Thus the assumption that FTHs do not contribute to the phenotype in these conditions may be misleading (Bassett & Williams 2008).

Bassett and Williams have published prolifically on thyroid-bone biology and have led on the counter debate regarding TSHR signalling in bone. Their work has explored the skeletal effects in mice of knocking out TRα, then TRβ, then both simultaneously to disrupt the HPT axis. This indicated T3 via TRα as the primary TFT parameter mediating bone effects. They observed that despite an excess of TSH(x400), T4(x15) in mice homozygous for a dominant negative TRβ mutation; the skeletal effects of thyrotoxicosis were not ameliorated by an extremely high TSH. Similarly the skeletal effects of hypothyroidism in TRα knockout mice was not ameliorated by normal TSH values (Kaneshige *et al* 2000, Bassett & Williams 2008).

Bassett *et al* (2008) compared skeletal phenotypes in genetically engineered CH mice (3 per group) *with* functional TSHRs but no thyroid ($Pax8^{-/-}$), versus no functional TSHRs ($Hyt^{-/-}$; T4 ~5% of normal); both had grossly elevated TSH. The skeletons of these mice were reported to be similar, and consistent with the expected effect of profound hypothyroidism. The skeletal abnormalities in $Hyt^{-/-}$ mice appeared to resolve completely when TRT was given (despite barely functional TSHRs), and the skeletons of heterozygous $Hyt^{-/+}$ mice (with relatively normal TFTs) appeared to be normal. The investigators went on to evaluate the effects of TSH *ex-vivo* on primary osteoblasts and osteoclasts and a cAMP response to TSH or TSI was not detected (in contrast to the aforementioned studies). Thus, this study suggests against TSHR having significant effects on bone physiology (Bassett *et al* 2008).

However, when critically appraising this paper I make the following observations: despite fairly marked differences in thyroid hormone levels between the groups (none in $Pax8^{-/-}$ versus low levels in $Hyt^{-/-}$) this did not appear to influence the skeletal phenotype despite there being no question over the influence of FTHs on bone. Similarly the $Hyt^{-/+}$ mice had less thyroid hormone than WT (4.8mg/dL +/- 0.7 versus 5.7mg/dL +/-0.8) albeit an insignificant difference for the low numbers evaluated, which again appears inconsequential. I propose that a higher TSH in these mice *might* counter balance an effect from lower T4 on bone. Clearly very low subject numbers limits the power of this study to identify differences. The authors focus on the similarities between the mouse skeletons, whereas differences such as the femoral bone densities (~18% less than WT in $Hyt^{-/-}$ versus ~6% less than WT in $Pax8^{-/-}$) are not considered to be important. Finally it is possible that the influence of TSHR activation in bone may be lost in the context of profoundly abnormal FTHs which does not exclude an effect in other circumstances. Nonetheless this work provides evidence *against* the notion that TSHR has direct effects on bone.

In reference to the Abe paper of 2003, Bassett and Williams offer a different interpretation. They suggest that the cause of low neonatal BMD in $TSHR^{-/-}$ mice is likely to relate to profound hypothyroidism at this crucial stage of development (rather than reduced TSHR signalling). The fact that the bone parameters did not normalise completely (in $TSHR^{-/-}$ mice) despite TRT at weaning (age 3 weeks) probably reflects the effects of neonatal hypothyroidism, and hypersensitivity to TRT thereafter. The reduced BMD in the heterozygous animals they feel

is overstated, being only ~6% below WT. However, this interpretation has been challenged; although neonatal hypothyroidism adversely affects bone quality (epiphyses and bone morphogenesis), a remodelling defect is not expected (Gothe *et al* 1999). Nonetheless their points are valid and correcting for these factors is very difficult.

Bassett and Williams' review article (2008) raises additional counter concerns regarding the TSHR - BMD notion. These include inconsistent data relaying TSH's effect on osteoblast differentiation (enhanced differentiation reported by Sampath *et al* (2007), suppressed differentiation described by Abe *et al* (2003) and absent effects observed in their studies (Bassett *et al* 2008)) and the fact that TSI in GD does not appear to protect against thyrotoxicosis-related bone loss. However, the clinical studies required to determine the influence (if any) from TSI on bone requires adequate subject numbers (with GD versus NTD), appropriate matching (age, health, thyroid function (grade & duration), treatment modalities and subsequent TFTs) and a sufficient period of observation to confidently determine or exclude BMD differences. Unfortunately such studies are nearly impossible to perform.

Table 1.7 summarises several studies that have attempted to differentiate bone effects in subjects with and without GD. It can be seen that In general differences are not seen.

There are many human studies that do not suggest independent TSH - bone associations. Several of these studies are summarised in table 1.8.

In regard to making sense of these discrepant reports it is important to remember that hormones can have different effects in different environments. Therefore discrepant effects in different environments (i.e. thyroid status related, gender or age-related, duration dependent etc) is not a reason to disregard an association. However, the information displayed in tables 1.4-1.8 demonstrate the ongoing uncertainties relating to the TSH - bone relationship.

Table 1.7: Studies comparing bone parameters in subjects with Graves' disease versus nodular thyroid disease.

Paper	Summary of study	Key observation
Jódar *et al* 1997	Evaluated BMD in a heterogeneous group (regards age, sex, severity & duration) with current (n=78) or previous (n=49) hyperthyroidism and healthy controls (n=43). Aetiology of hyperthyroidism was NTD in 25 and GD in 102 subjects.	A generalised decrease in BMD was evident in the spine of hyperthyroid subjects that improved (not normalised) with treatment. No BMD differences related to aetiology of hyperthyroidism.
Senturk *et al* (2003)	Evaluated osteotrophic cytokines; Interleukin 1, 6 & 8 and TNFα in patients with hyperthyroidism; 14 GD, and 36 NTD (versus 30 euthyroid controls).	Cytokines were elevated in hyperthyroidism with no differences relating to aetiology.
Amato *et al* 2004	Evaluated osteoprotegerin levels in hyperthyroid subjects (GD; 93, NTD; 21) pre and post correction with methimazole versus 68 age and sex matched controls.	Osteoprotegerin levels associated with FTHs and normalised more slowly with treatment in GD than NTD. At 1 year (study end) BTMs were similar in treated subjects and controls. No differences related to the aetiology of hyperthyroidism.
Antonelli *et al* 2006	Evaluated CXCL10 (an interferon-inducible chemokine associated with osteoclast differentiation (Grassi *et al* 2003, Lee *et al* 2012)) in subjects with hyperthyroidism (33 GD, versus 11 NTD), pre and post methimazole treatment. Also evaluated in 11 thyroidectomised subjects (relating to thyroid cancer), on TRT.	CXCL10 levels were higher in GD (than NTD despite similar TFTs) and dropped in GD on methimazole. Administration of rhTSH to the thyroidectomised subjects did not affect levels. They concluded that CXCL10 was associated with autoimmunity and did not relate to TSHR activation.
El Hadidy *et al* 2011	Evaluated BT and BMD in 52 men with treated hyperthyroidism; 31 had GD and 21 had NTD. They were compared against healthy, euthyroid, age-matched controls (n=25).	Hyperthyroid-treated subjects had similar BT and BMD, irrespective of cause. Their BMD was lower than controls. BT correlated negatively with TSH and positively with FTHs.

Abbreviations for tables 1.7 and 1.8 are as follows: GD; Graves' disease, NTD; Nodular thyroid disease, BTMs; Bone turnover markers. rhTSH; recombinant TSH, ♂; Male, ♀; Female, TRT; thyroxine replacement therapy.

Table 1.8: Studies that do not identify independent TSH-bone associations.

Paper	Summary of study	Key observation
Gürlek & Gedik 1999	Evaluated BT and BMD (at lumbar spine) in 15 premenopausal ♀ with Shyper (TSH<0.1) against age- and build-matched euthyroid controls.	No differences in BMD or BT parameters apparent between the groups.
Heijckmann *et al* 2005	Evaluated BMD (spine and hip) in subjects thyroidectomised (relating to thyroid cancer), on TRT with TSH <0.05 (n=59). Their DXA Z-scores were compared against the NHANES reference group.	BMD was not significantly different in the Shyper group versus the reference cohort.
Reverter *et al* 2005	Evaluated BMD in thyroidectomised subjects (relating to thyroid cancer) on TRT with Shyper (n=88) versus healthy euthyroid age-matched controls (n=88).	BMD was not lower in the Shyper group versus controls.
Giusti *et al* 2007	Evaluated BTMs in response to rhTSH in thyroidectomised subjects with a history of thyroid cancer (n=24; 7♂, 7 pre-menopausal ♀, and 10 post-menopausal ♀).	BTMs (osteoprotegerin and RANKL) did not alter in response to rhTSH administration.
Papadimitriou *et al* 2007	Evaluated BMD and BTM on ♂child siblings with CH (on TRT) due to a TSH-β mutation causing low TSH.	BMDs and BTMs were within the normal reference range for age.
Roef *et al* 2011	Evaluated BMD and TFTs in healthy ♂s aged 25-45 years (n=677).	An inverse association between FTHs (within the normal range) and BMD was seen but no BMD-TSH associations.
Di mase *et al* 2012	Evaluated BMD in 36 SH children and adolescents with untreated SH versus 25 age- and sex-matched healthy euthyroid controls.	BMD was not significantly different between the groups.
Marawaha *et al* 2012	Examined BMD and TFTs in 1290 Indian subjects <50 years old (1115 euthyroid, 175 with SH). The participants had no history of overt thyroid dysfunction.	No associations between TSH and BMD was revealed.
Van Rijin *et al* 2014	Evaluated peri-menopausal Dutch ♀s with no history of thyroid or bone disorders (n=1477). Subjects with SH, TPO antibodies or aberrant T4 were excluded.	An inverse independent association between T4 and BMD was seen but no TSH-BMD associations.

1.11 TSHR and Fat

When considering fat cell biology it is important to be aware of the differences between white, brown (BAT) and orbital fat. Whilst brown and white fat are derived from MSCs, orbital fat is derived from neuroectoderm (Billon *et al* 2008). In addition 'beige' adipocytes are recognised (that behave like BAT given appropriate stressors (prevalent in the inguinal regions of rodents (Harms & Seale 2013)). Orbital fat resembles BAT in appearance and certain behaviours (Joel 1965). Combs *et al* (2004) report that mice rendered hyperadiponectinaemic developed expanded interscapular BAT and exophthalmos indicating a common response from both depots. The fact that hyper- and hypothyroidism have effects on fat mass (and temperature regulation) and GO (relating to TSI) precipitates pathological orbital adipogenesis suggests that TSHR activation affects adipose tissue.

Rodbell (1964) reported TSH responsiveness from *non-thyroid* tissue after investigating the effect of TSH on rat adipocytes (testicular fat pad); concluding that it triggered lipolysis. Winand & Kohn (1972) postulated the presence of a "response element" in the retro-orbit (of guinea pigs) responsive to TSH and an "exophthalmogenic factor" (from the sera of GO subjects; likely to represent TSI). Both enhanced ATPase activity which they surmised would be relevant to GO pathogenesis (indicating lipolysis occurring via the TSHR-cAMP-PKA pathway (de Lloyd *et al* 2010)). Roselli-Rehfuss *et al* suggested the presence of TSHR in white and BAT based on the extraction of TSHR mRNA (messenger RNA) from these sources. They proceeded to propose a role for adipocyte TSHR in thermogenesis (Roselli-Rehfuss *et al* 1992).

Crisp *et al* (1997) from our unit demonstrated TSHR mRNA in *human* fat using Northern blotting analysis (and RT-PCR). It was described to be at the limit of Northern blot detection in omental and subcutaneous fat, but readily detectable in GO orbit (as with TG). They surmised that increased preadipocyte numbers in GO orbit may explain this observation. TSHR expression is recognised to be upregulated in fat depots undergoing adipogenesis (Crisp *et al* 2000, Haraguchi *et al* 1996), reflected by increased TSHR transcript numbers (Valyasevi *et al* 1999, Starkey *et al* 2003); a phenomenon exaggerated in GO. Starkey *et al* 2003 from our unit evaluated TSHR transcription rates in hyperthyroid and hypothyroid mouse fat depots.

Hyper- and hypothyroidism did not affect TSHR transcription rates despite being aggravating factors for GO (Walsh *et al* 1999).

A study evaluating TSH effects on adipocytes from preterm human infants (or fetuses) revealed a lipolytic effect (not evident in older subjects in whom catecholamines are the primary mediator of lipolysis) (Marcus *et al* 1988, Janson *et al* 1995). This observation might explain the physiological TSH surge, in neonates, in the first days of life (Oddie *et al* 1978). At this stage a lipolytic TSH effect to generate energy substrate, would be beneficial. Indeed, neonatal adipocytes respond poorly to catecholamines (potentially reflected by a high α2: α1 adrenoceptor ratio; α2 inhibits lipolysis (Marcus *et al* 1993, Elgadia *et al* 2010).

Elgadia *et al* (2010) generated and investigated adipocytes from TSHR knockout mouse pups (in reality *knockdowns*). Their adipocytes were 10 times less responsive to TSH-induced lipolysis (at physiological concentrations) than controls (although catecholamine-induced lipolysis remained normal). Their adipocytes were larger and basal lipolysis rates higher, though similar if corrected for surface area. They concluded that TSHR expression affects adipocyte function although its role was unclear.

Endo and Kobayashi (2012) compared mouse fat stores in PH (through thyroidectomy) *versus* $Hyt^{-/-}$ mice (with non-functional TSHRs). They noted proportionally reduced epididymal fat mass and adipocyte size in the thyroidectomised mice. They then introduced functional TSHR into the $Hyt^{-/-}$ mouse's unilateral fat pad (via plasmid injection and electroporation) and observed a 60% fall in fat mass compared with the control side (method performed using empty plasmid). Lipase mRNA expression doubled in the TSHR-transfected fat pad. They concluded that TSHR has a lipolytic role in the white fat of hypothyroid mice (Endo & Kobayashi 2012).

By contrast Jiang *et al* (2015) report suppression of triglyceride lipase (TL) by TSH. They evaluated adipocyte behaviour in TSHR knockouts (on TRT) versus WT mice, and investigated, in vitro, using the 3T3L1 cell line. TSHR knockout adipocytes had substantially reduced TL relative to controls, and TL expression increased in the 3T3L1 cells during differentiation, but fell in mature cells in response to TSH.

Several groups suggest a role for TSHR in BAT thermogenesis. Endo and Kobayashi (2008) reported that $Hyt^{-/-}$ mice were profoundly hypothermic in cold conditions, despite TRT. However, when functional TSHRs were introduced into their BAT, their cold intolerance improved. It is unclear if this observation is relevant to humans where neonatal BAT represents ~5% of their birth weight (Lean *et al* 1986) and declines with age. However, positron emission tomography reveals small amounts of BAT in most adults in mediastinal, interscapular and cervical regions (Virtanen *et al* 2009 & Ouellet *et al* 2012). In rodents, BAT has a role in non-shivering thermogenesis (BAT contains mitochondria expressing uncoupling protein (UCP1); enabling metabolic energy to dissipate as heat (Nicholls 1983)). If this effect could be reproduced in human BAT, then an anti-obesity effect would be anticipated.

Zhang *et al* (2006) from our unit manipulated GO pre-adipocytes to express a constitutively active TSHR (TSHR*). These cells demonstrated increased early adipocyte differentiation markers and UCP1 expression, but reduced late differentiation markers and the cells were refractory to PPARγ induced adipogenesis. A role for TSHR in the differentiation of pre-adipocytes into BAT was suggested.

TSHR expression is known to increase during adipogenesis. Zhang *et al* (2009a) from our unit evaluated the effect of TSHR* on the mouse 3T3L1 cell line against cells expressing GSP (activated Gsα) or transfected WT TSHR. GSP cells showed a reduction in PPARγ1 (non-tissue specific), undetectable PPARγ2 (fat-specific isoform required for adipogenesis) and unmeasurable phosphorylated FOXO1 (*phosphorylated* FOXO1 is ubiquinated and degraded, whilst FOX01 is a transcription factor that represses adipogenesis from PPARγ promotors (Armoni *et al* 2006). The effect of TSHR* was intermediate between GSP and WT cells suggesting that Gsβγ signalling (via P13K) may be ameliorating these effects. They concluded that Gsα signalling appeared to be suppressing adipogenesis in TSHR-expressing preadipocytes (Zhang *et al* 2009a).

This observation could suggest that up-regulated TSHR expression during adipogenesis might serve to moderate adipogenesis. In which case up-regulated TSHR expression in GO might be a protective response to pathological adipogenesis. However, this hypothesis does not fit in with the observation that raised TSH in PH/SH aggravates GO.

Chen *et al* (2015) reported a reduction of fatty acid synthase (FAS) in mature adipocytes in response to TSHR signalling (PKA and ERK mediated). Whilst TSHR expression and FAS were upregulated in obese mouse adipocytes. They suggested that TSHR effects may alter in obesity.

Lu and Lin (2008) explored the effect of (supraphysiological) TSH concentrations on mouse embryonic stem cell differentiation. TSH appeared to accelerate the terminal stages of adipogenesis, even in the absence of adipogenic factors. It also enhanced TSHR expression on differentiation day 12. This effect appeared to be PKA pathway mediated. They concluded that TSH was an adipogenic factor (Lu & Lin 2008).

Kumar *et al* (2011) support this conclusion and report that TSI enhanced GO preadipocyte to adipocyte differentiation (in adipogenic medium). They found that the P13K inhibitor hindered adipogenesis suggesting the effect was via the TSH-Gβγ-P13K pathway. This group suggest that the contrasting observations from Zhang *et al* 2009 may relate to the transfection process itself.

Lu *et al* (2012) evaluated TSHR expression in obese mice and humans (versus lean). They observed increased TSHR expression (not corrected for fat mass) in obese mice, and an increased TSHR/actin ratio in obese humans relative to lean. They also reported hindered adipogenesis in TSHR knockout 3T3L1 cells (versus controls) and concluded that TSHR expression was altered in obesity, and it may contribute to this process.

In conclusion, there is ample data indicating that TSHR signalling influences adipocyte function. It appears that its effects depend on the cell or organism, its differentiation potential or stage of development, its source (depot and origin), as well as its ambient environment. However, at present the functional significance of TSHR signalling in adipocyte biology remains uncertain.

1.12 TSHR and Muscle

Hyper- and hypothyroidism can precipitate pathological changes in muscle tissue. This can range from subtle muscular symptoms to florid signs associated with histological abnormalities (i.e. myopathies, pseudohypertrophy). Although skeletal muscle is most commonly affected, cardiac and extra-ocular muscle injury (in GO) occur.

In GO the extra-ocular muscles are typically oedematous, inflamed and the surrounding connective tissues expanded (by glycosaminoglycans such as hyaluronan and chondroitin sulfate (Hansen *et al* 1999). However, the myocytes generally remain intact (Hufnagel *et al* 1984) until fibrotic transformation occurs late in the disease (Campbell 1989).

Crisp *et al* (1997) from our unit investigated the presence of TSHR in human skeletal muscle; firstly via mRNA extraction and RT-PCR, and secondly via Northern blotting. In neither method was TSHR demonstrated. These results were consistent with subsequent reports evaluating abdominal skeletal muscle (Busuttil & Frauman 2001, Paschke *et al* 1993).

Drvota *et al* (1995) published data supporting the presence of TSHR in human heart. This was revealed through mRNA extraction and RT-PCR and confirmaed by Northern blotting. In addition, a TSH-induced cAMP response from mouse AT-1 cardiomyocyte cells was reported. However, subsequent work from Busuttil and Frauman (2001) did not support these findings (evaluating 5 human heart samples). They suggested that previous reports (Koshiyama *et al* 1996 and Sellitti *et al* 1997) were 'erroneous', and a likely consequence of contamination (from fat/connective tissue) and/or "illegitimate transcription" (i.e ubiquitous and minimal genomic transcription in all cells, not of functional significance (Chelly *et al* 1981)). Huang *et al* (2014) challenge this opinion by reporting the presence of functional TSHR in rodent ventricular myocytes (revealed by RT-PCR and various immunochemistry techniques). They report upregulation of myocyte-associated mRNA transcripts (HMGCR and β-MHC) *ex-vivo*, in H9c2 cells in response to TSH. In addition, rodent TSHR knockout ventricular cells expressed less HMGCR and β-MHC mRNA than WT.

TSHR is reported to be expressed on vascular smooth muscle cells (VSMC) (Sellitti *et al* 2000). Tian *et al* (2014) stated that TSHR stimulation induced VSMC proliferation and enhanced

progression through the cell cycle (determined by flow cytometry). They suggest that this observation may be relevant to reports of increased vascular dysfunction and atherosclerosis in SH.

Kloprogge *et al* (2005) evaluated extra-ocular muscle immunohistochemically and reported the presence of TSHR *within* and between myocytes. However, subsequent investigations using *in situ* hybridisation techniques restricted this to the *perimysial fibroblasts* within muscles (Spitzweg *et al* 1997). TSHR-expressing fibroblasts were also identified in non-GO orbit but to a lesser extent.

Boschi *et al* (2005) evaluated TSHR expression immunohistochemically in extra-ocular muscle comparing GO (n=30) and non-GO samples (n=20). They reported TSHR expression on fibroblast-like cells between myocytes in GO samples only. The TSHR expressing fibroblasts were more prevalent in early disease, and their levels correlated with serum TSI (around diagnosis). They concluded that TSHR-expressing fibroblasts may contribute to the pathogenesis of GO myocyte injury.

Subsequent investigation by our group revealed that preadipocyte fibroblasts manipulated to express TSHR* respond by increasing hyaluronan synthase expression. They suggested that this observation might explain the increase in subcutaneous hyaluronan observed in thyroid diseases (diffuse in PH, and more focal in GD (i.e. pretibial myxoedema and GO (Zhang *et al* 2009b)).

Smith (2015) suggest that TSHR-expressing orbital fibroblasts may seed to the orbit haematogenously and derive from the HSC-monocyte lineage. This theory relates to their CD34 expression, and their ability to synthesise inflammatory cytokines (given appropriate stimulation (i.e. TSHR and IGF-1)).

In conclusion; it is likely that the muscular dysfunction commonly accompanying thyroid disorders is caused by altered FTH levels predominantly. However, it is likely that perimyseal TSHR-expressing fibroblasts contribute to inflammatory injury in their vicinity, particularly in GO. Although the majority of studies do not demonstrate TSHR expression on myocytes themselves, the data is inconsistent and thus uncertainty remains.

1.13 Hypothesis generation and aims

The TSHR is expressed in a range of tissue types that includes those relevant to build (namely in bone, fat and muscle). Its functional significance in these locations is uncertain although there is mounting evidence to suggest an independent effect from TSHR activation, particularly in bone. TFT-component and TSH associations are frequently assessed in SH cohorts (in relation to parameters of interest) enabling cause-effect relationships to be proposed.

I have outlined a range of aetiologies encompassing SH that include genetic causes. The prevalence of inactivating TSHR-M carriers within adult cohorts are rarely determined. As these subjects have inherently reduced TSHR signalling capacity they are a useful group to study to explore for potential TSHR signalling-related effects. Subjects with TSHR-Ms and other genetic causes for SH are expected to reside in non-autoimmune cohorts. Thus subjects with and without thyroid autoimmunity are compared in this study to explore potential aetiology-specific effects.

My hypothesis was that the clinical implications of SH are aetiology dependent, and that TSHR signalling affects bone physiology.

The primary objectives of this SH study were as follows:

- To determine the prevalence of TSHR-Ms in an adult cohort.
- To examine whether TSHR-M status correlates with bone parameters (BMD or BT).
- To examine bone parameters in TPO antibody positive versus negative subjects.
- To evaluate bone parameters alongside individual TFT components, with a particular interest in TSH associations.

The secondary objectives of this study were to explore metabolic-risk parameters (adiposity, blood pressure, lipids, insulin resistance) in relation to TFT components, TSHR-M status and gene polymorphisms (TSHR and FOXE1 polyalanine tract length (PTL)), and TPO antibody status. These study objectives are discussed in their dedicated results chapters, and all

objectives, with their associated observations are provided in the concluding and closing chapter.

This study is the largest of its type to assess the prevalence of TSHR-Ms in an adult SH cohort and the 1st to compare bone parameters in this subgroup (relating this to TSHR signalling) versus the cohort. It is also the 1st study to determine FOXE1 PTL polymorphism status in a dedicated SH cohort. As such this project is unique in design and is expected to bring helpful clinical data to the SH literature and to provide direction for future research.

CHAPTER 2: METHODS

2.1 Recruitment and Participation

Patients with treatment naïve de novo SH (an elevated TSH and a T4 in the normal reference range) and aged between 18 and 70 years (at the time of the screening TFT) were recruited to the study as follows. Colleagues in the department of biochemistry flagged up and reviewed the clinical information provided on the biochemistry request forms of all potentially suitable subects. In those whose information did not meet our exclusion criteria (section 2.1.2), General Practitioner (GP) letters were prepared (Appendix 2; Letter of Approach to GP). This letter summarised the SH studies aims and objectives, provided their patient's identification details and TFT results, and included a GP reply slip. A patient information sheet was also enclosed, to be passed from the GP to subjects suitable for the study (Appendix 3). The GP reply slip advised; the subject's suitability for the study, their willingness to participate, and whether the GP had granted us permission to approach their patient about the study.

I collected and processed all reply slips and proceeded to contact all seemingly suitable and willing subjects (for which permission to approach the patient was granted). After confirming suitability for the study, I offered and arranged study participation to all eligible subjects.

Participation involved two visits to the hospital. The first was an 8am *fasting* appointment to the Clinical Research Facility (CRF), UHW. The 2nd was to the Medical Physics department, UHW for a DXA scan.

At the CRF appointment after I'd obtained the subjects official consent to study participation (section 2.2), I recorded their medical history, anthropometric data and collected and processed their blood and urine samples.

Subsequently I fed back the clinical results from the study to the participant and their GP, with clinical recommendations where indicated.

2.1.1 Study inclusion criteria

- Male or female subjects aged between 18 and 70 years (on the screening bloods).
- Subjects with treatment naieve de novo SH (as demonstrated by a normal free T4 and elevated TSH (of any degree)).
- Subjects for whom written informed consent to participate in the study had been obtained, prior to any study related activity.

2.1.2 Study exclusion criteria

- Subjects aged <18 or >70 years (on screening bloods)
- Pregnancy and breastfeeding
- Pre-existing thyroid disease treated with surgery, radioiodine therapy or drugs (carbimazole, propylthiouracil, thyroxine or tri-iodothyronine)
- Metabolic bone disease: hypo/hyperparathyroidism, vitamin D deficiency, Paget's disease
- Osteoporosis
- Endocrine disease affecting BMD: Hyperprolactinaemia, Cushing's syndrome, Acromegaly, hypopituitarism (of any degree), hypogonadism
- Coeliac disease
- Malabsorption
- Anorexia nervosa
- Chronic renal failure
- Chronic liver disease
- Cystic Fibrosis
- Inflammatory bowel disease
- Currently or recent (within 6 months of study entry) exposure to: glucocorticoids, androgens, antiandrogens, glitazones, cyclosporin, chemotherapy, anticonvulsants, vitamin D or depot contraceptive preparations. Women taking oral contraceptives or oestrogen replacement therapy were eligible to participate but this information was recorded (for potential adjustment in the final multivariate analyses).
- Previous treatment with bisphosphonates, raloxifene, strontium or recombinant PTH

2.1.3 Predetermined power calculation

The study protocol (Appendix 1; Original Study Protocol) describes the original study design (section 4) with its associated power calculation (section 5). The original primary objectives for the study were to compare bone parameters in the TSHR mutation subjects against the TPO positive subjects. At that time it was believed that those with genetic causes of SH would reside exclusively within the TPO negative cohort, whereas the TPO positive subject's aetiology would be thyroid autoimmunity.

The data management assumptions used in the power calculation were as follows;

- A local audit suggested that ~250 de novo SH subjects would be identified per annum by the department of biochemistry
- A 70% participation rate was anticipated and for recruitment to run over a 2 year period
- 2/3^{rds} of the cohort were anticipated to be TPO antibody positive
- TSHR mutations were expected to affect up to 40% of TPO negative subjects (Alberti *et al* 2002).

The statistical predictions enabling 80% power with significance (α) set at 5% were as follows;

- A cohort of ~350 subjects would be recruited
- TSHR mutations would be identified in ~50 subjects
- The TPO positive group would comprise ~250 subjects
- A Z-score standard deviation of ~0.44 was predicted

However, the actual study differed from this design in several respects. All study participants were screened for TSHR sequence variance irrespective of their TPO status (to test the theory that they would reside exclusively in the seronegative cohort). In contrast to the original study design; the TSHR sequence variants were compared with their normal TSHR sequence counterparts (see chapter 4 section 4.5 and 4.5.1) and the TPO positive subjects were compared against their TPO negative counterparts (see chapter 4 section 4.4.4 to 4.4.6). This change in the study design limits the utility of the predetermined power calculation.

2.2 Consent

All study participants were provided with written documentation summarising the studies objectives and explaining what participation would involve (also explaining that their clinical, biochemical and genetic data would be used anonymously for this study (and related research in this academic unit (Appendix 3)). This information was re-affirmed verbally prior to written consent being obtained (Appendix 4).

2.3 Participant History

This included a past medical history, medication list, family history (including osteoporosis and thyroid diseases), a social history that included occupation and lifestyle (smoking and alcohol history and usage, and usual activity levels). For women a menstrual and pregnancy history were recorded (see Appendix 5).

2.4 Blood Pressure

Blood pressure (BP) was measured using an automated and appropriately calibrated OMRON HEM-CR19 sphygmomanometer (OMRON Healthcare UK Ltd, Milton Keynes, MK15 0DG). Subjects were positioned in a seated position with their back and arm supported (Pickering *et al* 2005). The cuff was positioned at ~mid-sternal level directly over skin, and inflated after ≥5 minutes at rest (Pickering *et al* 2005). BP was taken 3 times (as recommended by the British Hypertension Society (O'Brien *et al* 2002), with the average value used in subsequent analyses. If one of the readings was discrepant, this was discarded and a further reading taken.

2.5 Height

Subjects were measured without shoes, with head, heel and buttocks touching the Seca 217 mechanical stadiometer (Supplier Seca UK, Birmingham, B5 5QB). Subjects stood with feet together, and head positioned so that their ear canal was level with their check bone. The stadiometer gauge was lowered on to their head (ensuring hair was flat) and a deep breath taken (protocol consistent with the PhenX Tool Kit (2014)). The gauge indicated their height, which was recorded to the nearest 0.5cms

2.6 Waist and hip circumference

This was measured at minimal respiration in an upright position using a flexible anthropometric tape measure (recorded to the nearest 0.5cms). For waist circumference this was positioned parallel to the floor between the iliac crest and the 12th rib posteriorly. The tape measure was taut, but not indenting the skin.

Hip circumference was measured at the point of maximal girth across the hips and buttocks (over thin, well fitted underpants) with the tape measure parallel to the floor.

Waist to Hip ratio was a simple calculation of waist circumference divided by hip circumference, recorded to 2 decimal points.

2.7 Weight and BMI

Subjects were weighed in light clothes (removing shoes, jackets, belts and emptying pockets) on a standard sit-on weighing scale (Seca 955) calibrated daily. Weight was recorded to the nearest 0.1 kilograms.

Body mass index (BMI) was recorded using the standard calculation of weight (in kg) divided by height (in metres) squared.

2.8 Percentage body fat

A Tanita TBF-538 fat monitor/scale (Tanita Corporation, Itabashi-Ku, Tokyo, Japan T174) was the bioimpedance meter used to measure 'Percentage body fat (%BF)'. The device was pre-programmed to 'adult' and the subject's sex and height (in metres) entered. The subject then stood barefoot and still on the device's metal foot plates. After ~30 seconds a %BF score was displayed and recorded to the nearest 0.5 units.

2.9 Blood sampling

A tourniquet was placed on the upper arm and the venepuncture site cleaned with an alcohol wipe, prior to venepuncture. Vacutainer bottles were filled directly, including 2 EDTA (ethylenediaminetetraacetic acid) bottles, four SST (serum separating tubes) bottles and one Fluoride Oxalate bottle (~25ml of blood in total). Thereafter the tourniquet was removed, needle withdrawn, and a sterile cotton wool ball placed over the puncture site with firm pressure applied for 2 minutes prior to a plaster being placed.

2.10 Urine collection

Participants were provided with a plastic container for midstream urine collection. Approximately 20ml of fresh urine was aliquoted from this pot to a universal container for storage at minus 20^{o}C.

2.11 Storing blood samples

The SST vacutainer bottles were placed in the Thermo Fisher Scientific Inc centrifuge in the CRF (Heraeus Megafuge 16R) soon after blood was taken. They were spun at 4000rpm for 15 minutes at minus 20^{o}C. Thereafter 0.4ml aliquots of serum were pipetted into appropriately labelled Eppendorf tubes; 3 were sent to the University Hospital of Wales (UHW) biochemistry laboratory for immediate analysis and those remaining placed in storage at minus 20^{o}C for subsequent analysis. Whole blood in EDTA vacutainers was placed directly into storage at 4^{o}C.

2.12 Extraction of DNA from whole blood

This method was in accordance with the manufacturer's instructions for QIAamp QIAGEN midikit (manufacturer Qiagen, Manchester, UK).

One hundred µl of Qiagen protease was added to 1ml of blood in a 15ml centrifuge tube and mixed. 1.2ml of buffer AL was added and mixed thoroughly before leaving to stand at 70^{o}C for ≥10minutes. 1ml of 100% alcohol was added and mixed thoroughly before the lysate was transferred to the midi-column and placed in the centrifuge tube provided. This was spun at 3000rpm for 3 minutes and the filtrate discarded. The midi-column was replaced in the dried

centrifuge tube and 2ml of buffer AW1 added to the column before spinning at 5000rpm for a minute. 2ml of buffer AW2 was added to the midi-column and centrifuged at 5000rpm for 15 minutes before the tube and filtrate were discarded. The midi-column was replaced in a clean centrifuge tube and 200µl of buffer AE pipetted onto the membrane of the midi-column, left to stand for 5 minutes, then centrifuged at 5000rpm for 2 minutes. This step was repeated. The filtrate of extracted DNA was tested for purity and concentration with a spectrophotometer (Amersham Pharmacia Biotech) prior to being placing in storage at minus 20^{o}C. On the spectrophotometer an absorbance 260:280nm ratio of 1.8 indicates pure DNA, and absorbance (at 260nm) of 1.0 equates to DNA concentration of 50 µg/ml.

2.13 TSHR departmental DNA primers for PCR amplification

The dHPLC screening tool (section 2.20.7 for details) was the initial test used to look for DNA sequence variation in a subject's TSHR exon. When this was suggested the exon concerned was PCR amplified (2.14), purified (2.15 & 2.17) and sequenced (2.16) for validation (2.18).

To genotype the TSHR I used previously optimised primer pairs covering all 10 TSHR exons and intron/exon boundaries. Their sequences, annealing temperatures and respective PCR amplicon sizes are detailed in table 2.1.

Table 2.1: TSHR exon-specific primer sequences, their annealing temperatures and respective amplicon sizes.

Exon	5'-3' sequence	Annealing Temperature (°C)	Fragment size (base pairs)
1F 1R	GAG GAT GGA GAA ATA GCC CCG AG CAC TAC TTC GGG CTG TTA TTG AG	54	302
2F 2R	TAA GGT GAA TTA TTA GAA AAG C CTT GAT AGA ACA CGT TTA GAG A	48	205
3F 3R	GCA GAA TCC ATC AGG GTT GT AGA AAC CAG GCC TCC CAT TG	54	304
4F 4R	ACC CTG TGG CGT AAA TGC ATA T CCC GAC CCA GGC TAT ACA CCA TT	52	329
5F 5R	GCT TTA CTT ATC TTC AAC CTA CC AGT TTG ACT ACA GGT TGT CTT C	52	291
6F 6R	TAT TGT GTC CTG TTA TTT AAG TGC ATA GTA CTC TTA GAG TAT ATA TGA TAA GG	56	293
7F 7R	TGG GAT ACA TAT GTG GGA CCT G TGT TGG GTC ACA CTA ACT CTG G	54	324
8F 8R	TGG TCA CAT TTT ATT CTG ATA TTT GT CTC CCC TTA ATG TCT CCA TTT ATT CC	54	272
9F 9R	TCA TCT CCC AAT TAA CCT CAG G GCT TCC AAT TTC CTC TCC AC	54	408
10aF 10aR	TGG CAC TGA CTC TTT TCT GT GTC CAT GGG CAG GCA GAT AC	56	868
10bF 10bR	ACT GTC TTT GCA AGC GAG TT GTG TCA TGG GAT TGG AAT GC	56	875

F; forward primer, R; reverse primer. 10a and 10b represent 2 overlapping primer pairs for the larger 10th exon, all others are represented by single primer pairs.

2.14 Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

PCR was performed using *Promega* reagents (Manufacturer Wisconsin, USA). Nuclease-free PCR tubes were prepared on ice, each contained 1µl (at 10pM/µl) of forward and reverse primer, 1µl of dNTP (10mM), 5µl of 5X buffer, 2µl of Taq polymerase (5U/ul) and ~100ng of subject DNA template (~2-4µl depending on concentration). This was made up to a 25µl final volume with nuclease-free water, vortexed and spun briefly prior to transfer to the thermocycler.

The PCR program was as follows: Initial denaturation at 95^{o}C x 3 minutes, then 30 thermocycles of [95^{o}C x 30 seconds, 30 seconds at primer pair-specific annealing temperature (Table M.1), 72^{o}C x 1 minute], then 72^{o}C x 10 minutes for the final extension, returning to a 4^{o}C hold on completion. In negative controls, water replaced genomic DNA.

A 2% agarose gel was prepared (1.5g Agarose powder boiled with 75ml of TAE buffer* (details below), then mixed with 3.75µl of ethidium bromide (EB; 0.5µg/ml). When solidified, this was placed in an electrophoresis tank with TAE buffer and EB at equivalent concentration. Samples were prepared for agarose gel wells as follows: 8µl of PCR product (~125ng) mixed with 2µl of EB, whilst the ladder comprised 2µl of ladder (0.13µg/µl), 2µl of EB and 8 µl of sterile water. Samples were pipetted into their respective wells and the electrophoresis current run for 30 minutes at 120 volts. TSHR amplicons were separated by agarose gel electrophoresis and their respective sizes estimated relative to a 100bp ladder (Promega; Cat no. G210A). The gel was inspected under UV light to confirm the presence of DNA bands of appropriate size. [**Tris-acetate-EDTA (TAE) electrophoresis buffer, prepared as a 50X stock solution and diluted to 1X (40mM Tris pH 7.6, 20mM Acetic acid, 1mM EDTA) with deionised water*].

2.15 Polethylene glycol (PEG) precipitation

The PCR product was purified using PEG precipitation. An equal volume of PEG solution (26% (w/v) PEG 8000/70mM $MgCl_2$) to PCR product (i.e. 20µl to 20µl) was mixed in Eppendorf tubes and left to stand for 10 minutes. Samples were spun at 13000rpm for 30 minutes and the supernatant removed. Five hundred µl of 70% alcohol was added, mixed thoroughly for a minute then centrifuged at 13,000rpm for 10 minutes. The supernatant was removed and the tube left to dry (~10 minutes). The pellet was then re-suspended in 20-40µl of nuclease-free water. After confirmation of DNA product the sample was stored at minus 20^{o}C.

2.16 DNA sequencing preparation

The BigDye Terminator sequencing Kit v3.1 (manufacturer *Applied Biosystems* Thermo Fisher Scientific) was used to generate single stranded DNA sequences in preparation for automated DNA sequencing (see 2.20.6).

Reagents were prepared on ice using nuclease-free PCR tubes. Into each tube I aliquoted 5µl of PEG precipitated DNA sample (~100ng), 1 µl of primer (forward or reverse at 10pM/µl), 2 µl of nuclease-free water and 2 µl of BigDye Mix.

The Sequencing PCR thermocycling programme was as follows: 1 cycle at 96^{o}C for 3 minutes, then 30 cycles [96^{o}C for 30 seconds and then 60^{o}C for 4 minutes], returning to a 4^{o}C hold upon completion.

2.17 Sodium Acetate

The PCR sequencing product was precipitated with Sodium Acetate as follows, prior to automated sequencing.

One µl of 3M Sodium Acetate was added to 10µl of post-PCR sequencing reaction and vortexed thoroughly; 30µl of 100% alcohol was added to the mix and left to stand for 10 minutes. This was centrifuged at 13,000rpm for 30minutes, the supernatant removed, and the tube left to dry. Five hundred µl of 70% alcohol was added to the tube, vortexed for a minute then centrifuged at 13,000rpm for 10 minutes. The supernatant was removed and the tube left to dry ~10 minutes before being stored at minus 20^{o}C or transferred directly to the sequencing laboratory (see 2.20.6).

2.18 DNA sequence interpretation

The National Centre for Biotechnology Information (NCBI) software was used to compare study subject TSHR exon sequence against standard TSHR sequence (from GENE ATLAS). This was performed using their Basic Local Alignment search Tool (BLAST) (see 2.20.6 for an example).

When BLAST identified a sequence nucleotide discrepancy the electropherogram was inspected at the equivalent codon position for verification. In addition the electropherogram was inspected along its length as a heterozygous nucleotide change (with duplicate nucleotide peak) can be missed by BLAST. Possible variants (that would change the translated amino acid) were confirmed by repeating the sequencing process using the reverse primer (to confirm the nucleotide sequence change at that location), thus enabling identification of TSHR polymorphisms or mutations.

2.19 Statistical Analyses

I used Microsoft Excel software to prepare data for subsequent analysis in Minitab 16 statistical programme (also used to create graphs).

The tests used to compare one data set against another were the *unpaired student t-test* (for continuous data) or '*proportion*' tests (for categorical data). The relationship between two continuous parameters was assessed using *basic regression analysis*. However, the majority of my analyses were more complex and for this I used *general linear multivariate regression analyses* and *stepwise multivariate regression analysis* (see section 4.4). In keeping with the condition of these tests, outlier results were removed and when the data did not follow a normal distribution in raw form, it was appropriately converted (log transformed or square rooted) to ensure this condition was met prior to its application.

In my analysis a p-value below 0.05 (a <1/20 probability of getting this result through chance alone) defined the threshold at which I rejected the null hypothesis and accepted the alternative hypothesis. The null hypothesis states that there is no difference revealed by the analysis other than that expected as a consequence of normal variation alone.

The 95% confidence interval (C.I) I provide alongside my analyses describe the range of values (lower to upper limit) that correlate with this level of confidence. The true value for the population mean (as opposed to the cohort) is expected to lie within the C.I in 95% of samples evaluated (with a 95% C.I). The C.I is calculated as the regression coefficient +/- 1.96 x the Standard Error (SE), with SE representing the sampling distribution's standard deviation (SD).

The regression coefficient (R) provided in my regression analyses (beside the predictor variable assessed) indicates (for significant results) the mean change in the response variable (y) for a one unit change in the predictor variable (in a positive (+) or negative (-) direction). The R^2 value (range 0-100%) provided alongside each regression analysis indicates the percentage of the response variable's variation (R^2 = explained variation / total variation) explained by a linear model.

2.20 Methods in collaboration with others

2.20.1 DXA bone Scan

BMD was evaluated by dual energy X-ray absorptiometry (DXA) using a Hologic Discovery Bone Densitometer (QDR series) and Apex Software (version 3.3.0.1). The lumbar spine (LS) (L1, L2, L3 and L4 and a total value) and left hip (femoral neck, greater trochanter, inter-trochanter and a total value) were assessed. The machine was checked daily against a Hologic spine phantom, and accommodates weights up to 159kg. Participants whose torso depth was >30cm were scanned on 'array' mode; all others were on 'fast' mode.

Participants were positioned supine on the scanner table. To evaluate the LS the patient's calves were supported on a box to position their femur at ~90^{o} to the spine, thereby flattening the lumbar lordosis. With optimum positioning the spine was straight, perpendicular to the scanning field, and the laser positioned over mid-L5. The region of interest was then scanned in to the software system for evaluation.

To evaluate the hip, the Hologic Hip positioning fixture was positioned between the patient's feet in the midline. The left leg was abducted and externally rotated before being internally rotated and adducted, securing the foot, via a sling, to the positioning fixture (internally rotating the leg at ~25^{o}). Optimum positioning held the shaft of the femur perpendicular to the scanning field revealing very little of the lesser trochanter.

Participant data was compared against normal population reference data of Caucasian, Oriental or Afro-Caribbean ethnic origins to generate T- and Z-scores. These data were provided by the NHANES study (Kelly *et al* 2009) for hip, and Hologic-specific datasets for LS.

The total radiation dose for the DXA scan was estimated at 11µSv (~2 days of UK background radiation exposure).

2.20.2 Automated biochemistry laboratory analyses

These analyses were performed in the Department of Biochemistry and Immunology, UHW. All analyses were performed in accordance with the manufacturer's recommendations (detailed in the operator's manual) and the Laboratory's *Standard Operating Instructions*.

These methods are briefly summarised in tables 2.2 and 2.3 (providing thyroid-related, and non-thyroid related analyses (bone profile, lipids and glucose) respectively). However, the detailed methods can be retrieved locally (laboratory reference file numbers are provided).

LDL-Cholesterol (LDL-C) was the only biochemistry parameter estimated and not measured (using the Friedewald formula applicable to fasting samples (Friedewald *et al* 1972) and shown below). This equation was applied to all suitable specimens, and the LDL-C results were automatically uploaded onto the laboratory software system (in mmol/L).

LDL-cholesterol = Total cholesterol - HDL-cholesterol - Triglyceride/2.2

The equation is not applicable with triglycerides >4.5 mmol/L, or with chylomicrons present. Its accuracy is reduced with an estimated LDL <1.8mmol/L and with high triglycerides (Martin *et al* 2013).

2.20.3 Managing the change to the thyrotrophin analyser

The TFT analyser changed mid- recruitment from the Siemens ADVIA Centeur immunoassay to the Abbott Architect i2000SR system. This occurred after the 139th subject had been recruited.

Comparative results from both thyrotrophin immunoassays indicated that the Siemens Centeur's assay results minus 10% provided an acceptable prediction of the Abbott Architect's assay results (see Appendix 8). These data, together with the conversion recommendation were provided by colleagues in the department of Biochemistry, UHW.

Equivalent evaluations on the T4, T3 and TPO antibody results indicated that they were not inter-convertible. Therefore these analyses were all run on the Abbott Architect assay; subject numbers <140 from stored serum, and numbers >139 on the day of their study participation.

This limitation to the study is discussed in section 6.2.

2.20.4 Urine Iodine

An Inductively Coupled Plasma Mass Spectrometer (ICP-MS) for urine iodine quantification was developed in the department of Biochemistry, Immunology and Toxicology, UHW supporting an MSc project. The optimum diluent and concentrations for the procedure were determined, and the method validated prior to its use on the SH study participants (Jones *et al* 2013).

It was performed on an Agilent 7700x ICP-MS with ASX 500 Auto-sampler and integrated sample introduction system (Agilent Technologies, Wokingham, UK) in the Trace Element laboratory. Argon gas for ICP-MS plasma and helium gas for ICP-MS collision cell were supplied by BOC (Cardiff, UK).

Iodine standards were prepared by diluting iodine standard 1000 µg/ml (Inorganic Ventures, Esslab, Hadleigh, UK) in ultrapure water (Standards 0, 10, 100, 500, 1000 µg/L).
The assay was validated on three in house urine pools, and monitored through commercial quality control and external quality assurance (EQA) via the Centres for Disease Control and Prevention EQUIP (Ensuring the Quality of Urinary Iodine Procedures) Programme (Atlanta, US).

Urine creatinine was measured on the Abbott Architect c16000 clinical chemistry analyser (Abbott Laboratories Ltd, Maidenhead, UK) and results expressed as urine iodine: creatinine ratio. The reference range was taken from Sieniawska *et al* 2012, and the cohort's Iodine status categorised according to the World Health Organisation's recommendations (World Health Organisation 2007a*).*

2.20.5 Analyses outsourced to specialist laboratories

This related to the Insulin and Bone Turnover Marker assays. Basic details relating to these assays are provided in table 2.5.

Table 2.2: Thyroid-related biochemistry analyses performed at UHW

Parameter evaluated, Manufacturer (Units)	Brief Method (Laboratory reference file)	Specifications		
		Normal Reference range	Analytical range	Precision and Accuracy
TSH Siemens (mu/L)	Sandwich immunoassay involving acridinium labelled anti-TSH mouse antibody and anti-TSH sheep antibody bound to paramagnetic particles. (LP-BIO-ETSH rev1 (2007))	0.35-5.0	<0.02; >150	5.87% at 0.75, 4.22% at 5.65 & 3.17% at 19mU/L
TSH Abbott (mIU/L)	CMIA involving antiβ-TSH antibody-coated microparticles and acridinium labelled antiα-TSH antibodies. Solutions are added to precipitate a chemiluminescent reaction. (LP-BIO-ETSH rev1 (2011))	0.35-4.5	<0.05; 100 (up to 1000 with dilution)	<10%
TPO antibodies Abbott (IU/ml)	CMIA involving TPO coated paramagnetic microparticles, acridinium labelled anti-human IgG and solutions to trigger a chemiluminescent reaction. (LP-BIO-AATPO rev1 (2011)).	<6	0-1000	<10% if >5.6IU/ml
T4 Abbott (pmol/L)	Competitive CMIA involving anti-T4 labelled paramagnetic microparticles, T3-acridinium labelled conjugate and solutions to trigger a chemiluminescence reaction. (LP-BIO-AFT4 rev1 (2011))	9-19.1	<5.15; >77.22	<10% between ~8.4 and 36 pmol/L
T3 Abbott (pmol/L)	Competitive CMIA; equivalent to the T4 assay above but substituting anti-T3 for anti-T4 labelled paramagnetic microparticles. (LP-BIO-AFT3 rev1 (2011))	2.6-5.7	<2; >46.08	<10%

The automated analysers were Abbott Architect i2000SR for Abbott (supplier Berkshire SL6 4XF) and the ADVIA Centaur Immunoassay Analyser for Siemens (supplier Camberley, Surrey, UK GU16 8QD). Abbreviations; Chemiluminescent Microparticle Immunoassay (CMIA).

Table 2.3: Non-thyroid biochemistry analyses performed at UHW.

Parameter assessed, (Units)	Brief Method (Laboratory reference file)	Specifications		
		Normal Reference range	Analytical range	Precision and Accuracy
Calcium (mmol/L)	Calcium reacts with a chemical (Arsenazo-III), in acid, producing an indigo complex measured at 660nm. (LP-BIO-ALCa rev1 (2007))	2.20-2.60	0.03-6.52	1.7% at 2.3 & 1.1% at 3.48mmol/L
Phosphate (mmol/L)	Ammonium Molybdate reacts with Phosphate To produce a coloured complex measured at 340nm. (LP-BIO-ALPO4 rev1 (2007))	0.8-1.45	0.2-817	1.1% at 1.03 & 0.5% at 2.5mmol/L
ALP (IU/L)	ALP catalyses hydrolysis of colourless p-nitrophenyl phosphate to yellow phosphate and n-nitrophenyl (at alkaline pH) measured at 404nm. (LP-BIO-ALAlkP rev2 (2007))	30-115	measurable to 22,775	3.2% at 94 & 2.5% at 417IU/L
Total Cholesterol (mmol/L)	Cholesterol oxidation produces H_2O_2 as a by-product. Peroxide combines with reagents (Hydroxybenzoic acid (HBA) and 4-aminoantipyrene) to form a chromophore measured at 500nm. (LP-BIO-ALChol rev1 (2007))	Not provided	0.26-18.26	1.6% at 3.36 & 0.8% at 6.95mmol/L
HDL Cholesterol (mmol/L)	Phase 1 removes non-HDL cholesterol (using DSBmT (N-bis-(4-sulphobutyl)-m-toluidine-disodium) producing colourless product. Phase 2 dissolves HDL-C in detergent that reacts with a chromogenic coupler and enzyme, producing a blue product measured at 604nm. (LP-BIO-ALHDLUlt rev1 (2007))	0.7-1.7	0.13-4.66 (without dilution)	5.5% at 0.54 & 1.4% at 2.04mmol/L
Triglyceride (mmol/L)	Tg is hydrolysed releasing glycerol. Glycerol is phosphorylated to glycerol-3-phosphate which is oxidised with H_2O_2 as a by-product. Peroxide reacts with a chemical (4-aminoantipyrene and 4-chlorophenol) producing red product, measured at 500nm. (LP-BIO-ALTrig rev1 (2007))	0.6 - 2	0.07 - 16.05 (without dilution)	1.1% at 1.2 & 0.8% at 2.13mmol/L
Glucose Abbott (mmol/L)	Glucose is phosphorylated to G6P. Oxidation of G6P reduces NADP to NADPH whose absorbance is measured at 340nm. (LP-BIO-ALGluc rev2 (2007))	3-6	to 44.4 (without dilution)	2.2% at 4 & 1.5% at 15.6mmol/L

All analyses were performed on the Abbot Aeroset analyser that measures absorbance spectrophotometrically (Abbott Diagnostics; supplier Berkshire SL6 4XF). Abbreviations; Glucose-6-phosphate (G6P), Nicotinamide Adenine Dinucleotide Phosphate (NADP), Alkaline Phosphatase (ALP), High Density Lipoprotein (HDL), Cholesterol (-C), Triglyceride (Tg).

Table 2.4: Urine Iodine assay specifications

Specification	Value
Intra-assay and Inter-assay precision	1.7-3.6%; 1.1-3.0%
Limit of quantification (CV <20%)	1.0 µg/L (8 nmol/L)
Reporting limit	5 µg/L (39 nmol/L)

Details provided by Miss Katie Jones, MSc Biochemist, UHW

Table 2.5: Insulin and Bone Turnover Marker assays

Parameter assessed, (Units)	Brief Method	Specifications		
		Normal Reference range	Analytical Range	Precision, Sensitivity
Insulin (pmol/L)	Two site immunometric sandwich assay involving insulin monoclonal antibody bound to a microtitre plate and acridinium labelled liquid phase. Luminescence relates to insulin concentration.	Fasting; <174	20-800	<7.5%, 0.12pmol/L.
P1NP (µg/L)	Electrochemiluminescent sandwich immunoassay: Involves biotinylated analyte-specific antibody, Streptavidin-coated microparticles, and analyte-specific antibody labelled with ruthenium complex. The microparticles are magnetically captured on an electrode and current precipitates chemiluminescence that is translated into an analyte concentration.	♂: 20-76 PM♀: 19-69	20-600	<3%, 8µg/L
CTX (µg/L)		0.1- 0.5	0.2-1.5	<3%, 0.01µg/L

The insulin analysis was performed by Professor Luzio's Diabetes Research Group in Swansea University. The P1NP and CTX assays were performed by Professor Fraser's group in Norwich University, East Anglia. Abbreviations; Procollagen type 1 N-terminal propeptide (P1NP), C-terminal telopeptide of type 1 collagen (CTX). Pre-menopausal Females (PM♀), Males (♂).

2.20.6 Automated DNA sequencing

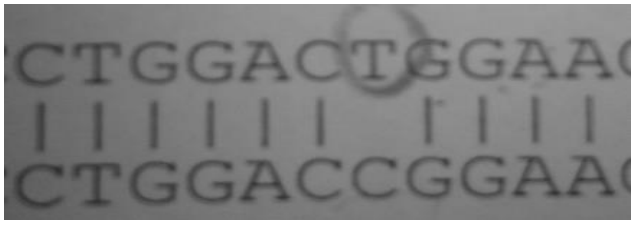
This was performed within the Biotechnology Services Department at UHW.

Samples were dried of residual ethanol and treated with highly deionised formamide prior to being run on the Applied Biosystems Genetic Analyzer (ABGA) 3130xl following its standard protocol.

The ABGA 3130xl used v3.0 Biosystems software for data collection, and v5.3.1 Biosystems software to convert raw sequence data into standard electropherograms. A PDF and printed electropherogram of the data were provided.

An example of a BLAST nucleotide alignment image (Figure 2.1) with its associated forward and reverse electropherograms (Figure 2.2) are shown below. This example reveals the heterozygous TSHR R531W mutation.

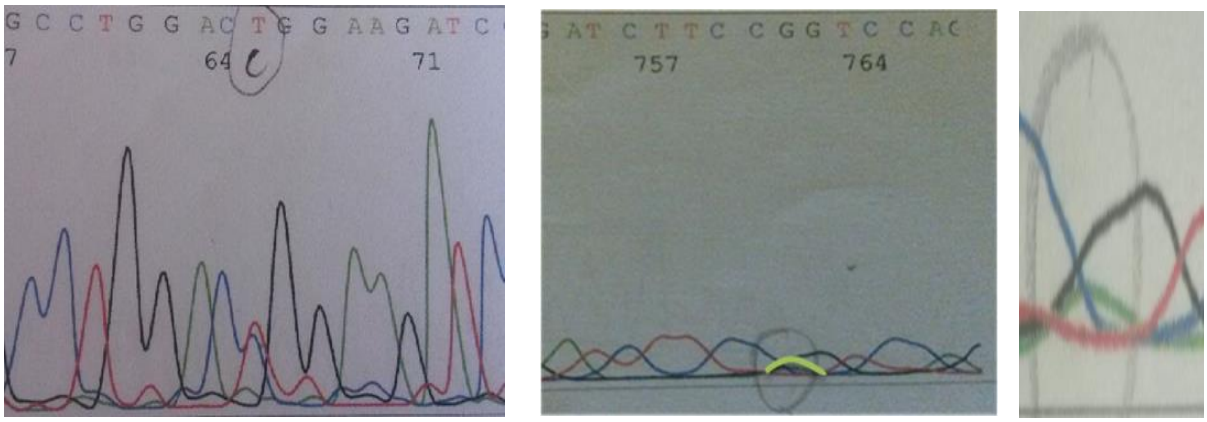
Figure 2.1: **Nucleotide alignment image**



O46 Sequence: CTGGAC**T**GGAA
Standard Sequence: CTGGAC**C**GGAA

Figure 2.2: **Electropherograms of the forward and reverse sequences for subject 046**

Forward Sequence | Reverse Sequence | Magnification



O46 Sequence:	CTGGAC**T**GGAA	TTCC**A**GTCCAG	C**A**G
Standard Sequence:	CTGGAC**C**GGAA	TTCC**G**GTCCAG	C**G**G

The duplicate peak on the forward sequence clearly reveals where T (Thymine) rather than C (Cytosine) is reported by the sequence scanner. The equivalent region on the reverse sequence is at the end of the sequence printout where quality is poor. Although the scanner reports a G (Guanine) in this position (consistent with standard sequence) careful inspection of the electropherogram reveals an A (Adenine) correlating with this heterozygous base change (circled in the reverse trace).

2.20.7 Denaturating High Performance Liquid Chromatography (dHPLC)

This work was commenced in the department of Immunology and Biochemistry (supporting PhD higher degrees) and completed by the thyroid research group, Cardiff University.

Thirteen TSHR dHPLC control fragments were designed (1 fragment for exons 1-9, and 4 overlapping fragments for the 10th exon) and the optimum melting temperature and buffer ratio (Buffer A (0.1M Triethylammonium Acetate [TEAA]) to Buffer B (0.1M TEAA and 25% acetonitrile)) determined for each dHPLC fragment's analysis. This information, along with the optimum melting temperature predicted by the dHPLC software system, was programmed into the Wave DNA Fragment Analyser System (Transgenomic Inc. Santa Clara USA). The dHPLC protocol was followed in accordance with the manufacturer's recommendations.

Study subject DNA was amplified using dHPLC fragment-specific primers (Tables 2.6 and 2.7). Thereafter, 20 µl of subject-specific PCR-amplified DNA was loaded into each well of the dHPLC machine (minimum of 5µl required per temperature specific programme). The machine has 196 wells reflecting the maximum number of simultaneous analyses that can be performed. A control sample per fragment assessed was run alongside all batched samples.

Table 2.6: Primer-specific annealing temperatures of dHPLC fragments

dHPLC Fragment	1	2	3, 5, 7, 10.1 & 10.4	4, 8, 10.2 & 10.3	6	9
Primer- specific annealing temperatures (°C)	65.4	55	61.4	57.8	55.1	61.4

dHPLC fragment numbers correlate with TSHR exon numbers with four fragments (10.1-10.4) for exon 10.

Elution profiles (chromatogram waveforms) were determined by the 'Wave Maker' (Transgenomic Inc software) and generated through a fluorescence (535nm) and ultraviolet detector (260nm). When subject-specific waveforms revealed heteroduplexes or waveforms substantially different from control (potentially suggesting heterozygous or homozygous mutations respectively), direct DNA sequencing of the subject's TSHR exon was undertaken.

Table 2.7: PCR programmes used per dHPLC fragment amplified.

Exon	PCR preparation	Initial phase of PCR programme	Later phase of PCR programme
1,2,3,4,7,9 & 10	2.5µl human DNA, 10.75µl H_2O, 1.5µl 25mM $MgCl_2$, 2.5µl 10x buffer, 2.5µl 2mM dNTP, 2.5µl forward primer 5µM, 2.5µl reverse primer 5µM and 0.25µl enzyme "Ampli Taq Gold DNA Polymerase" in a 25µl reaction.	95°C for 5mins **35 cycles** [95°C for 30 seconds, primer-specific annealing temperature for 1 minute, 72°C 1 minute] 72°C for 5 minutes.	95°C for 5 minutes **70 cycles** [95°C for 10 seconds, 60°C for 10 seconds]. **70cycles** [55°C for 10 seconds, 25°C for 10 seconds] Final hold 10°C.
5,6 & 8	2µl human DNA, 9.8µl H_2O, 2µl 10X buffer, 2 µl dNTP 2mM, 2µl forward primer 5µM, 2µl reverse primer 5µM and 0.2µl enzyme "HotStarTaq DNA Polymerase" in a 20µl reaction.	95°C for 15minutes **35 cycles** [95°C 40 seconds, primer-specific annealing temperature for 1 minute, 73°C 1 minute & 50 seconds] 72°C for 10 minutes.	

"Ampli Taq Gold DNA Polymerase" was provided by Applied Biosystems, Life Technologies, Paisley, UK.
"HotStarTaq DNA Polymerase" was provided by Qiagen, Manchester, UK.

2.20.8 FOXE1 polyalanine tract length (PTL) determination

This work was carried out by the thyroid research group, Cardiff University.

PCR FOXE1 primers were designed to cover a portion of the FOXE1 coding sequence and its polyalanine tract. The 5'-3' nucleotide sequences of these primers are shown below:

FOXE1 Forward: GCG GAG GAC ATG TTC GAG A

FOXE1 Reverse: CGC GGG GTA GTA GAC TGG AG

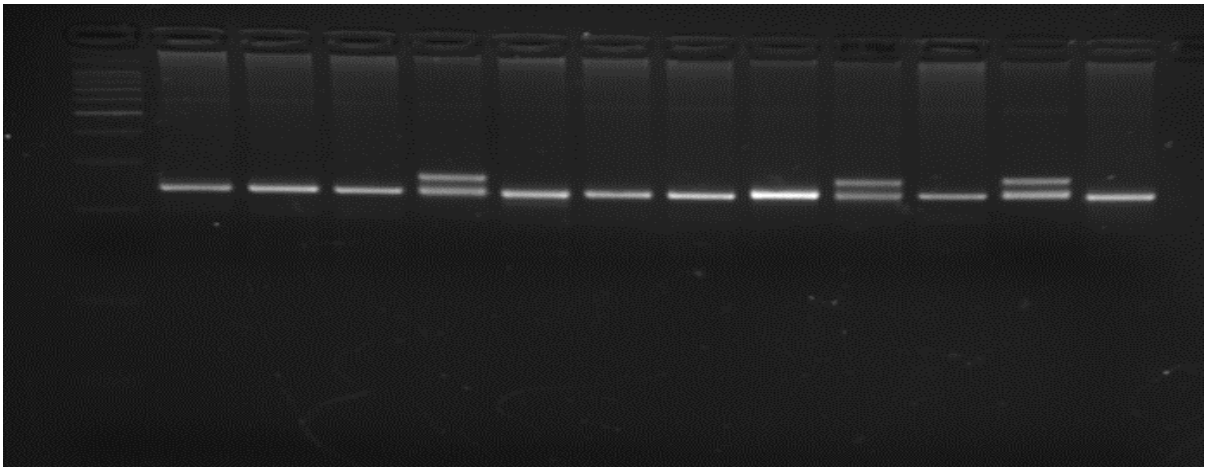
FOXE1 PCR was performed in nuclease-free PCR tubes; 2.5µl of 10X PCR buffer was added to a solution containing 5µl of Q-solution, 0.5µl (10mM) dNTP mix, 5µM forward primer (2 µl of 10pmole/µl), 5µM reverse primer (2µl of 10pmol/µL), 0.5 µl of TaKaRa Taq polymerase and 0.1-1.0 µg of DNA template. This was made up to a 25µl volume (with nuclease-free water), vortexed and spun briefly before transfer to the thermocycler.

The FOXE1 PCR program was as follows: initial denaturation at 95^{o}C X 1 minute, followed by 35 cycles [94^{o}C X 1 minute, 60^{o}C X 1 minute, 72^{o}C X 1 minute] then 72^{o}C X 10 minutes for the final extension before returning to a 4^{o}C hold.

The samples were run on a 4% agarose gel alongside a 100bp Qiagen ladder to determine their PTL status. This was confirmed by direct sequencing if required.

An image of a FOXE1 PTL polymorphism gel is shown below. This reveals heterozygosity (14/16) for samples in the 4th, 9th and 11th column from the ladder. All other samples are homozygous for the more prevalent FOXE1 14/14 PTL polymorphism.

Figure 2.3: FOXE1 PTL agarose gel image



Courtesy of Dr Ameen Bakhsh, thyroid research group, Cardiff University

CHAPTER 3: RECRUITMENT EXPERIENCE AND OBSERVATIONAL DATA INCLUDING THYROID FUNCTION TEST DATA AND GENETIC EVALUATIONS IN A SUBCLINICAL HYPOTHYROID COHORT

3.1 Introduction

Subclinical Hypothyroidism (SH) was first described as a clinical entity in 1973 (Hall & Evered 1973). The arbitrary values to define SH vary according to the regional normal range and the analytical assay used (Hollowell *et al* 2002, Casey *et al* 2005). However, they are generally based on the 95% percentiles of normal thus a TSH >97.5th percentile (raised) with a T4 >2.5th and <97.5th percentile (normal) define SH.

Highly sensitive TSH assays were introduced in the late 1980s and early 1990s and transformed the diagnostic utility of thyroid function testing (Toft 1988; Hay *et al* 1991). Thyroid function tests (TFTs) are now widely and routinely requested and there is a wealth of data correlating the TSH and T4 parameters to each other. This relationship was originally described as *inverse log linear*, but subsequently recognised to be more complex (Hoermann *et al* 2010). Hadlow *et al* (2013) recently described the relationship as a *non-linear inverse log with sigmoid shaped inflexion points at the extremes of T4* (in that case; 7 and 21pmol/L). Variations in the relationship according to sex and age are described (Clarke *et al* 2012, Hadlow *et al* 2013).

Free-T4 (T4) appears to be the main determinant of TSH levels through its negative feedback on the hypothalamus and pituitary (Fonseca *et al* 2013, Sawin *et al* 1977, Abend *et al* 1991, Introduction; figure 1.2). However its purpose appears to be the maintenance of normal free-T3 (T3) levels (Fonseca *et al* 2013). T3 is the active hormone with ~15x the affinity of T4 for the thyroid hormone receptors (Lin *et al* 1990). It is recognised that T3 drops acutely in severe illness (Economidou *et al* 2011) whereas T3 and/or T4 are elevated and TSH suppressed in primary hyperthyroidism (Dayan 2001). Outside these contexts the relationship between T3 versus TSH or free-T4 are rarely explored.

The majority of human SH studies are *observational* studies intent on revealing any adverse consequence from SH. Such studies reflect the epidemiology of SH particularly its female preponderance and increased prevalence amongst older subjects (Tunbridge *et al* 1977; Canaris *et al* 2000, Hollowell *et al* 2002). Other observations in SH include the following:

- SH is often transient, normalising on re-testing in between 25% (Parle *et al* 1991) and 50% of subjects (Diez & Iglesias 2009). For this reason it is recommended that TFTs are repeated 12 weeks after an initial SH result is obtained, to confirm the biochemical picture (Surks *et al* 2004).
- The prevalence of thyroid autoimmunity in SH in iodine replete regions ranges from ~40% (Surks & Hollowell 2007) to 80% (Allan *et al* 2000). However recently *iodine insufficiency* has been demonstrated in these regions (Vanderpump *et al* 2011). The prevalence in communities traditionally considered as *Iodine insufficient* is much less (Teng *et al* 2011) although exact figures are scant due to the limited health resources in these predominantly underdeveloped regions (Vanderpump 2011).
- SH is more likely to progress to hypothyroidism in subjects with higher TSH values and thyroid autoimmunity (annual progression rates in females (♀) of 4.3% (TPO +ve) versus 2.6% (TPO -ve), and higher again in TPO positive men (♂) (x4) according to the 20 year Whickham study data (Vanderpump *et al* 1995, Tunbridge *et al* 1977).
- SH is less common amongst people of African and Caribbean origins versus Caucasians, this is attributed to these races having an inherently lower TSH set-point (Hollowell *et al* 2002)
- SH is less common in smokers than non-smokers (Knudsen *et al* 2002).

A challenge for any clinical study is recruiting the *target number* of participants to enable the study objectives to be robustly evaluated. In studies such as mine that depend on General Practitioner (GP) participation; participation rates of ~80% may be expected (Roland *et al* 2001). Thereafter participation rates of ~35-50% initially (Cornelia *et al* 2005) with drop-out rates of ~≥20% (Bell *et al* 2013) are described. Insufficient recruitment reduces the power of a study to determine its objectives and increases the probability of misleading observations being made (Patel *et al* 2003). It is also difficult to recruit a representative sample i.e. women can be under-represented (Harris & Douglas 2000), language difficulties discourage ethnic

minorities, homeless subjects lack access to research, and *selection bias* is present when subject and/or GP choice determines participation (Patel *et al* 2003). Thus although the data obtained from such studies are assumed to reflect the characteristics of the population recruited, this may not be the case.

SH has many causes although autoimmune thyroid disease (ATD) is the most widely studied and most common in the UK (Vanderpump 2011). Relatively few studies have explored the genetic causes of SH, and where this has been done it has focused on paediatric cohorts. The prevalence of heterozygous TSHR mutations (TSHR-M) (and gene variants) has been explored in several Italian paediatric *non-autoimmune* SH cohorts. Camilot *et al* (2005) reported a prevalence of 11% (13/116) in their child and adolescent subjects; Calebiro *et al* (2012) reported 12% (18/153) in their group, whilst Nicoletti *et al* (2009) reported 30% (11/38) in their smaller group (aged 1 to 11 years). Alberti identified TSHR-Ms in 4 of 10 subjects with neonatal or juvenile non-autoimmune SH (TSHs 6.6-46mU/L), 8 of whom had familial SH (Alberti *et al* 2002). Adults were evaluated in a study where SH subjects and their SH relatives were screened for TSHR-Ms. TSHR gene aberrance was revealed in 5/42 (12%) samples, and all affected subjects had familial not sporadic SH (Tonacchera *et al* 2004). In our unit, two siblings with congenital hypothyroidism (CH) were diagnosed with homozygous inactivating TSHR-Ms secondary to the nonsense mutation W546X. Thereafter, blood from 368 individuals in the local South Wales area (184 healthy blood donors and 184 schizophrenic patients [thyroid function unknown]) were screened for the W546X mutation; two displayed W546X heterozygosity. This indicated a background prevalence of ~1/180 (Jordan *et al* 2003) suggesting that this TSHR-M may be relatively common in the South Wales population.

TSHR-Ms are believed to reside in subjects who lack thyroid autoimmunity (though exceptions are reported (Tonacchera *et al* 2001). However, the case finding process has generally evaluated seronegative subjects. Interestingly, it is suggested that 70-80% of the predisposition to ATD is genetically determined (Hansen *et al* 2006) and the TSHR has been implicated in genetic association studies examining the prevalence of ATDs, including hypothyroidism (Akamizu *et al* 2000). However, subsequent studies restricted the association to Graves' disease (GD) (Tomer 2010). TSHR polymorphisms (in coding and noncoding regions, and in close proximity to TSHR) appear to predispose to GD (Brand *et al* 2009, Stefan *et al*

2014) and Graves Orbitopathy (Jurecka-Lubieniecka *et al* 2014). Thus the assumption that TSHR gene variance will affect only the seronegative cohort may not be correct.

Common TSHR polymorphisms include the D727E, P52T and the D36H. The prevalence of D727E heterozygosity amongst control groups is variably reported at 10% (Matakidou *et al* 2004) to 16% (Mühlberg *et al* 2000), P52T heterozygosity is estimated at ~7% (Simanainen *et al* 1999) and the D36H at 0.5% to 5% (Peeters *et al* 2003, Simanainen *et al* 1999). The D727E polymorphism has been associated with marginally lower TSH levels in twin studies (Hansen *et al* 2006) and previously associated with increased TSHR sensitivity (Gabriel *et al* 1999). However, functional studies to support this statement were not reproduced (Nogueira *et al* 1999). The prevalence of this polymorphism has been explored in a range of thyroid conditions including neonatal SH (Teofoli *et al* 2007), GD (Ho *et al* 2003) and toxic nodular thyroid disease (NTD) (Mühlberg *et al* 2000). However, a consistent association with any of these conditions is lacking. Interestingly, subjects homozygous for this polymorphism have been identified incidentally in studies (rather than consequent to a disease process (Van der Deure *et al* 2008)), suggesting its functional significance, if any, may be small. The P52T and D36H are less widely studied but appear *not* to be associated with a disease entity (Ho *et al* 2003, Sunthornthepvarakul *et al* 1999) or altered TSHR sensitivity (Cuddify *et al* 1995, Gustavsson *et al* 1995).

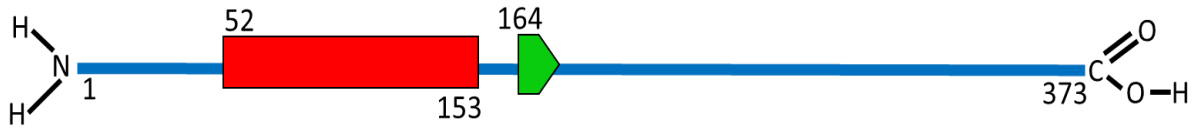
As explained throughout this thesis, one of my primary objectives was to explore bone mineral density (BMD) and bone turnover (BT) in relation to the aetiology of SH including TSHR-M status (see chapter 4). This was also explored in TSHR D727E polymorphism carriers. This objective relates to the observation that TSHR resides in extra-thyroidal environments including bone (reviewed in Davies *et al* 2002) and that TSHR knockdown status (Abe *et al* 2003, Baliram *et al* 2012) and even TSHR D727E polymorphism status (Van der Deure *et al* 2008, liu *et al* 2012) are reported to influence bone physiology and BMD. Thus identifying TSHR gene aberrations and exploring their associations with thyroid function parameters was the first step towards this objective.

One of my secondary objectives was to explore whether body composition parameters vary in relation to TSHR gene variance. This relates to the observation that TSHR is expressed on mesenchymal stem cells (Bagriacik *et al* 2012) with their ability to differentiate into distinct

terminal tissue types (including bone, fat and muscle; see figure 1.5). Thus TSHR activation might influence lineage specific differentiation and body composition (de Lloyd *et al* 2010). Given the association between adiposity and metabolic-risk parameters (Eckel *et al* 2014), (results chapter 5) this was something I wished to explore.

The FOXE1 gene (otherwise known as Thyroid Transcription Factor 2) is a member of the forkhead family of transcription factors that plays a vital role in thyroid gland morphogenesis. The gene is located on the long arm of chromosome 9 (9q22) (Chadwick *et al* 1997), has 1 exon and ~1/3rd of the gene is coding sequence (Venza *et al* 2011). The FOXE1 gene is expressed in a range of cells and tissues with mRNA extracted from liver, muscle, heart, brain and fat tissue, and the protein demonstrated in hair follicle outer root sheath (Brancaccio *et al* 2004, Bullock 2007) and testis (Genecards human gene database; FOXE1 (www.genecards.org)). FOXE1 influences the transcription of thyroglobulin and thyroid peroxidase (Francis-Lang *et al* 1992) and polymorphisms in the FOXE1 gene have been associated with altered thyroid hormone and TSH levels (Gudmundsson *et al* 2009, Taylor *et al* 2015). A schematic illustration of the protein product is shown below.

Figure 3.1: Illustration of the FOXE1 protein



The amino acid number from amino to carboxyl terminal is provided. The red box represents the DNA binding domain, and the green triangle indicates the location of the variable length of polyalanine tract (adapted from the atlas of genetics and cytogenetics in oncology and haematology (http:atlasgeneticsoncology.org/genes/FOXE1).

Homozygous mutations in FOXE1 cause Bamforth-Lazarus syndrome in humans, and an equivalent phenotype in mice (Baris *et al* 2006, De Felice *et al* 1998). This syndrome's features include CH (consequent to thyroid dysgenesis), cleft palate and spiky hair (Bamforth *et al* 1989).

The FOXE1 gene has in its makeup a polyalanine tract, with various length polymorphisms (PTL) in the tract (12, 14, 16, 17, 19 and 22) identified (Kallel *et al* 2010, Bullock *et al* 2012). The most prevalent genotype is the homozygous 14 PTL polymorphism affecting 50% of a

Slovenian control cohort (Watkins *et al* 2006) and 97% of a Japanese control cohort (Hishinuma *et al* 2001). Whilst the next most prevalent genotype is the heterozygous 14/16 carried in ~3% (Hishinuma *et al* 2001) to ~25% (Watkins *et al* 2006), according to the population studied. All other PTLs are uncommon. It has been shown that the 14 PTL polymorphism (or wild type) has higher transcriptional activity than the 16 PTL (Bullock *et al* 2012).

FOXE1 PTL polymorphisms have been explored in association with a range of thyroid disorders. The short PTLs (<14) have been implicated (on an infrequent basis) with thyroid dysgenesis (TD) (Hishinuma *et al* 2001). Carré *et al* (2007) genotyped 115 TD cases versus 129 controls and reported an association between the 14/14 genotype and TD, with the 14/16 (or 16/16) being protective. Thyroid cancer association studies have associated the 16/16 PTL with an increased risk of papillary thyroid cancer (x~2.5) versus the 14/14 (Kallel *et al* 2010, Bullock *et al* 2012). To date no association between FOXE1 PTL and ATDs have been made (Kallel *et al* 2010). However, in all studies evidence to support causation between the implicated FOXE1 PTL polymorphism and the thyroid disorder are lacking.

My study is unique in that the total SH cohort will be screened for TSHR gene variance, irrespective of TPO antibody status. This will help to determine whether mutations reside exclusively in the TPO seronegative patients or not. This is the largest study to date to screen SH subjects for TSHR-Ms, and the first to evaluate FOXE1 PTL polymorphisms in a dedicated SH cohort.

I will now proceed to describe my study recruitment experience and the observational data obtained from this cohort including subject characteristics, TFTs and genetic analyses.

3.1.1 Recruitment Experience

Recruitment ran over a 23 month period from January 2009 to December 2010, during which time 1537 GP letters were sent out to 85 GP practices feeding in to our biochemistry department, and to 8 outside our catchment area. 93% of feeder practices (79/85) responded to at least 1 letter of approach, versus 2 (20%) of GPs outside our catchment area. Three of 85 feeder practices expressed a wish to receive no further correspondence on the study.

In total, a response from GPs was received on 45% of occasions (692/1537) and their responses were as follows: granting permission to approach their patient in 67% (463/693); advising not suitable in 17% (116/693), advising that the patient was unwilling to participate in 13% (91/693), and in 3% (23/693) advising that the patient was no longer resident at their practice (see figure 3.2, and figure 3.3 that summarises my recruitment experience).

Of the 463 patients I was allowed to invite to the study; 220/463 (48%) were confirmed as willing and suitable, and appointments to participate were arranged. Of the others; in 13% (61/463) participation was declined, in 21% (98/463) they failed to respond to my letters or phone calls, in 15% (70/463) they were willing but unsuitable (included 7 with consent difficulties; 4 with minimal English language, and 3 with learning difficulties) and a further 3% (14/463) contacted the study after recruitment had closed.

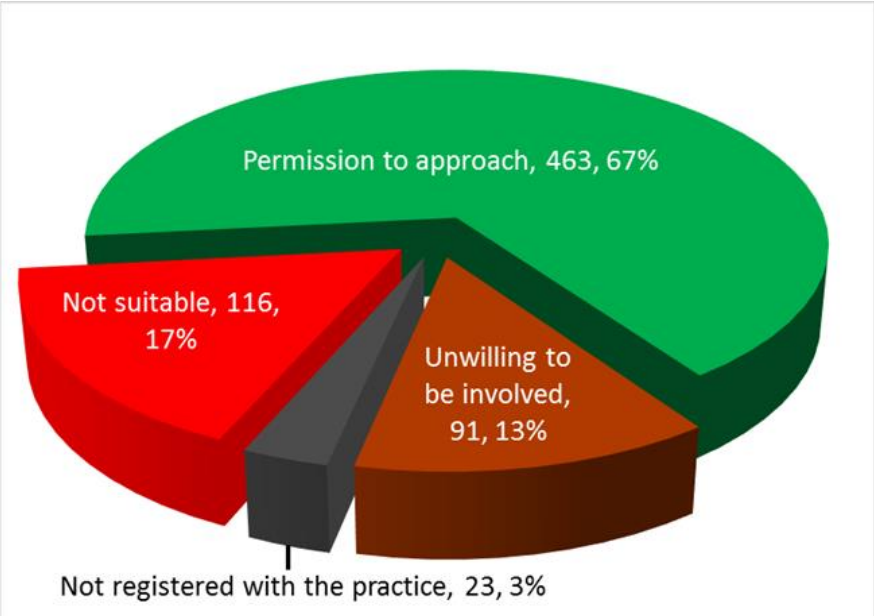
Of the 220 subjects booked in to the study, 12 (5%) repeatedly failed to attend appointments, 2 were found to be unsuitable at study attendance, and one chose not to be included in the study after attending the initial session.

Three subjects were recruited to the study through different avenues; 1 referred by a GP out of area (who we had not approached), and 2 (eligible hospital staff) referred themselves to the study.

The final study cohort consisted of 208 subjects representing ~14% of those originally identified by the department of biochemistry (208/1537).

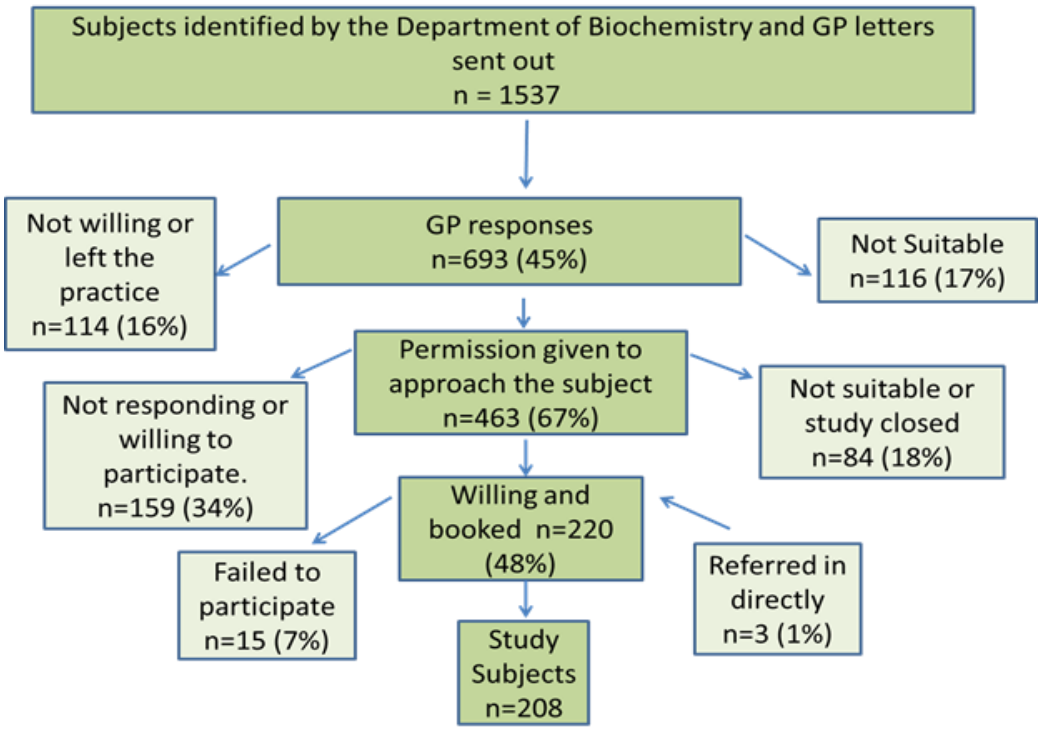
Of the 208 study participants; 7 (3%) did not have DXA scans (6 due to repeated failures to attend appointments, and 1 exceeded the machine's weight limit) and DXA scans were incomplete in a further 6 patients (one due to bilateral hip replacements, 4 due to spinal metalwork, and 1 due to a naval piercing).

Figure 3.2: Pie chart detailing GP responses to our letter of approach



The categories are as labelled with subject numbers and their percentage amongst the group provided

Figure 3.3: Flow diagram summarising my subject recruitment experience



The number of subjects (n) and the percentage (%) at each step are provided

3.1.2: Study participation in relation to gender and age

In this study gender did not influence study participation (Table 3.1).

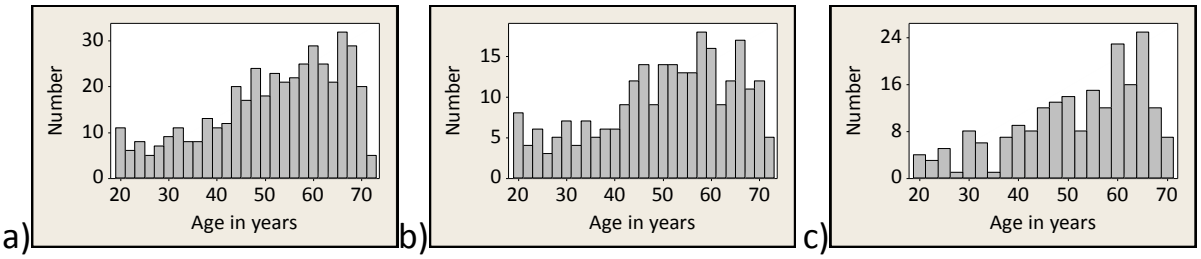
Table 3.1: Study participation observations in relation to gender and age

	Seemingly suitable for study	Not choosing to participate	Study Participants
Female ratio and %	(349/475), 73%	(189/254), 74%	(156/208), 75%
Mean, Median and Modal ages (Years)	51, 53, 65	50, 52, 57	51, 55, 61

The group 'Seemingly suitable for study' excluded those not registered with the GP, unsuitable for the study, and those with responses too late to participate. The group 'Not choosing to participate' included those unwilling to participate (from GP response, or my direct communication including those failing to respond).

Figure 3.4: Histograms illustrating the age distributions amongst subject groups

a) To approach about the study **b) Declining participation** **c) Study participants**



The number of subjects per age group are shown on the y axis and age in years is shown on the x axis. The relative distribution of subject ages across each group (a-c) is depicted in the individual graphs.

It is evident that the age distributions are broadly similar across each of the subgroups depicted in figure 3.4 (subjects to be approached about the study, those declining participation, and those participating in the study).

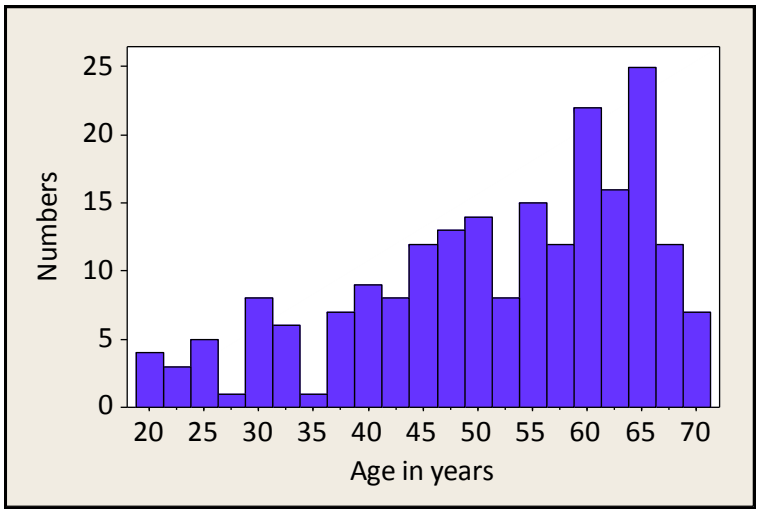
These data indicate that the recruited cohort provide a good representation of the greater cohort in terms of *age* and *gender ratios*.

3.2 Demographic and observational data on the SH cohort

3.2.1 Demographics

The study cohort was 75% female (156/208) and 90% Caucasian (9% Asian and 1% Afro-Caribbean/ Black). The mean age of participants was 51 years (median age 55) and age at study attendance ranged from 20 to 71 years (see figure 3.5 below).

Figure 3.5: Histogram of age distribution across the cohort



The histogram shows age on the x-axis and subject numbers on the y-axis.

3.2.2 Cohort comorbidities

- Hypertension; 30% (63/208) had this diagnosis.
- Diabetes affected 10% (20/208); One with type I and nineteen with type II diabetes (3 diet controlled, 5 on insulin therapy, and the others were on oral anti-hyperglycaemic agents). One subject was identified with probable undiagnosed diabetes on his study participation bloods.
- Vascular diseases; 5% (10/ 208) had a diagnosis of Ischaemic Heart Disease and 1% (3/208) a diagnosis of Cerebrovascular Disease.

3.2.3 Lifestyle characteristics and indicators

- Smoking history;

  30% (64/208) had a significant smoking history (which I define as a >10 pack year smoking history (equivalent to 20 cigarettes per day for *more* than 10 years) *or* a 10 pack year history *and* a current smoker).

  Of the total cohort; 13% (27/208) were current smokers, 33% (68/208) were ex-smokers, and 54% (113/208) had never smoked.

- Self-reported average alcohol intake;

  22% (46/208) were teetotal, 71% (147/208) drank within recommended limits and 7% (15/208) drank in excess of the 1995 Joint societies recommended limits; >21 units per week for men and >14 units per week for women (A working group of the Royal College of Physicians, Psychiatrists and General Practitioners (Marmot *et al* 1995)).

- Self-reported activity levels; ~50% (101/208) described their lifestyle as sedentary (which I defined as no exercise outside of basic activities of daily living) whilst 6% (13/208) described themselves as very fit (>150minutes of vigorous aerobic activity per week (Moore *et al* 2012).

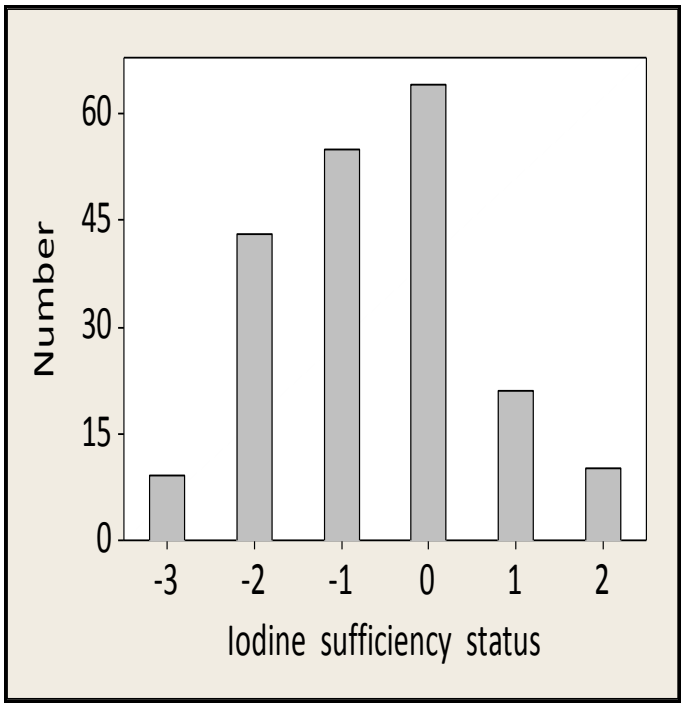
- BMI; 72% (150/208) were overweight or obese (according to the WHO classification sytem), (see figure 3.7).

- The cohort's iodine status was categorised as *mildly iodine deficient* (at 93 µg/l (range 8–3340 µg/l)

  Urine iodine excretion (as a marker of Iodine sufficiency) was evaluated and categorised according to *median* urine iodine excretion values in accordance with The World Health Organisations classification system (WHO 2007a); severe insufficiency <20 µg/l, moderate insufficiency 20-49 µg/l, mild insufficiency 50-99 µg/l, adequacy 100-199 µg/l, surplus 200-299 µg/l and excessive >300 µg/l.

I have described the Iodine sufficiency status of individuals within the cohort according to the WHO Iodine excretion thresholds (described) However their intended application is to cohort and population data only. The data is provided in figure 3.6.

Figure 3.6: Iodine sufficiency status of individuals within the SH cohort



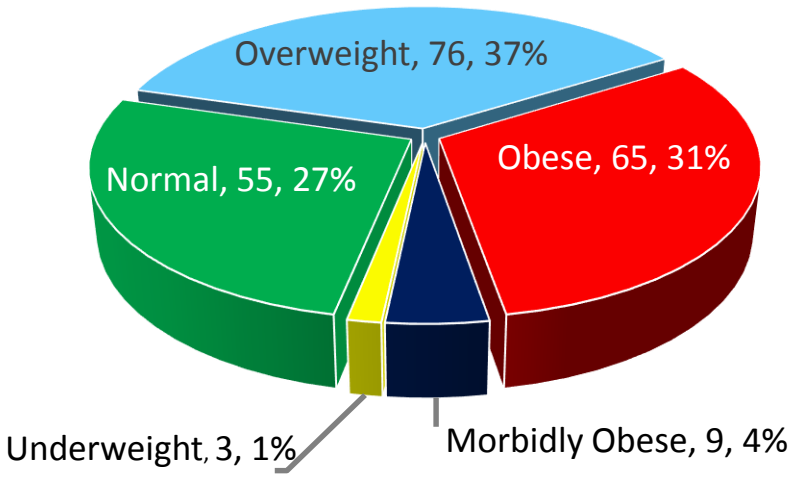
Iodine sufficiency status		Percentage of cohort
-3	Severely Insufficient	5%
-2	Moderately Insufficient	21%
-1	Mildly Insufficient	27%
0	Adequate	32%
1	Surplus	10%
2	Excessive	5%

Iodine sufficiency status was categorised according to each subjects urine iodine excretion as; severely insufficient <20 µg/l, moderately insufficient 20-49 µg/l, mildly insufficient 50-99 µg/l, adequate 100-199 µg/l, surplus 200-299 µg/l and excessive >300 µg/l. This information was available in 202 subjects.

These data suggest that aberrant iodine sufficiency status, particularly in the more extreme groups may explain or contribute to these subjects SH (Prete *et al* 2015).

However, an association between iodine status and TPO antibodies or TSH values was not revealed in this study.

Figure 3.7: Pie chart of BMI weight categories across the cohort



The categories are as labelled with their subject numbers and percentage amongst the group provided

BMI categories are in accordance with the WHO classification system;

- <18.5kg/m^2 *Underweight*
- 18.5-24.9kg/m^2 N*ormal* weight for Caucasians (18.5-22.9 for Asians)
- 25-29.9kg/m^2 O*verweight* for Caucasians (23-27.4 for Asians)
- 30-39.9kg/m^2 O*bese* for Caucasians (≥ 27.5 for Asians)
- ≥40kg/m^2 M*orbidly Obese*

3.3 TPO antibody status

Thyroid peroxidase (TPO) autoantibody status was classified as positive (+ve) or negative (-ve) according to the antibody titre (reference range <6 to >1000 IU/ml (Abbot2000i analyser). A titre <6 was considered -ve and above this threshold +ve (see Table 2.2 for assay details).

Half the study cohort were TPO antibody +ve and half -ve. In table 3.2 the cohort have been compared for a range of parameters, according to their TPO antibody status.

Table 3.2: Compares the TPO positive versus negative groups for the parameters listed

Variable	TPO +ve	TPO -ve	difference	95% C.I.	p-value
Sex (% Male)	17%	35%	-18%	-6% to -29%	0.003
$\bar{x}$ **Age (years)**	49.2	54.4	-5.15	-1.65 to -8.65	0.004
Thyroid Family History	53%	31%	+20%	+7% to +33%	0.004
$\bar{x}$ **TSH at study attendance**	5.81	4.79	+1.02	+0.34 to +1.7	0.002
$\bar{x}$ **T4 at study attendance**	12.71	13.6	-0.9	-1.29 to -0.5	<0.001
$\bar{x}$ **T3 at study attendance**	4.05	4.12	0.07	-0.08 to +0.21	0.35
$\bar{x}$ **log^{10} urine Iodine/ creatinine**	0.18	0.12	+0.07	-0.03 to +0.17	0.18

In table 3.2 $\bar{x}$ symbolises mean value, the difference between the TPO +ve and -ve groups are provided alongside the 95% confidence interval (C.I.) and p-value. The plus (+) and minus (-) signs indicate the direction of the difference. Units are as follows; T4 and T3; pmol/L, TSH; mU/L and Iodine /creatinine ratio; µg/L.

The differences between the TPO +ve versus -ve groups were as follows:

- A higher proportion of women (83% versus 65%)
- A lower mean age (49 versus 54 years)
- More frequently reported *thyroid family history* (FH) (53% versus 31%)
- Lower mean T4 (12.7 versus 13.6pmol/L) and higher mean TSH (5.8 versus 4.8mU/L)

T3 and Urine Iodine excretion were not significantly different between the groups.

3.4 Thyroid Function Tests in the SH cohort

3.4.1 Interval change in TSH across the cohort

The average time between the original TFTs being taken (that enabled study participation) and repeat TFTs being performed at study attendance was 11 weeks (range; 3-14).

The TSH fell during this time period in 77% (160/208), back into the reference range (<5mU/L) in 47% (96/208). However it remained ≥3mU/L in 90% (184/208) and above 2.5mU/L in 93% (193/208) (many experts consider TSH <2.5mU/L as a *true* normal value (Wartofsky & Dickey 2005). See figures 3.8-3.10.

Figure 3.8: Histogram illustrating the distribution of the original TSHs that enabled study participation

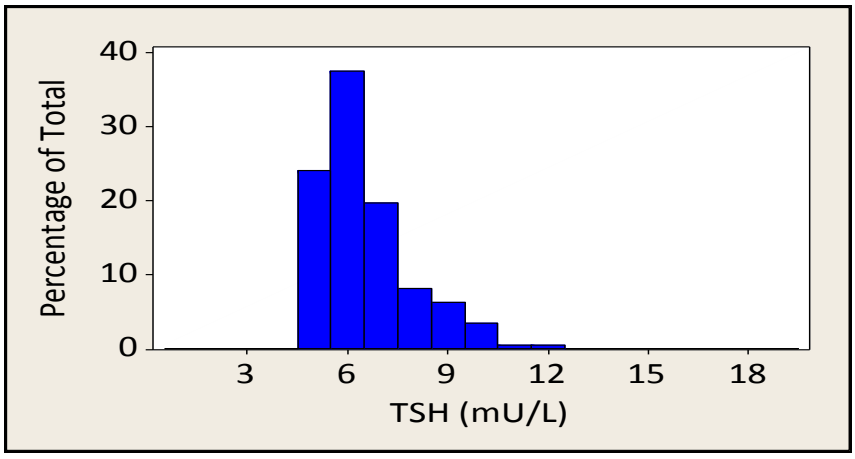


Figure 3.9: Histogram illustrating the distribution of subsequent TSHs taken at study participation

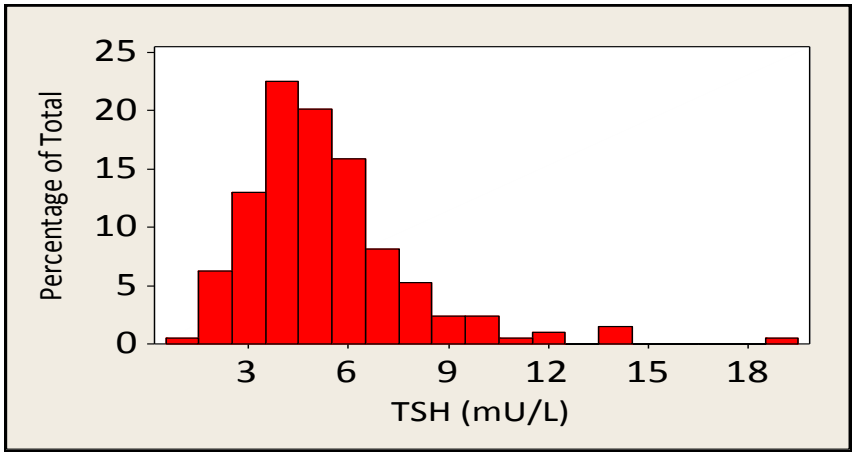
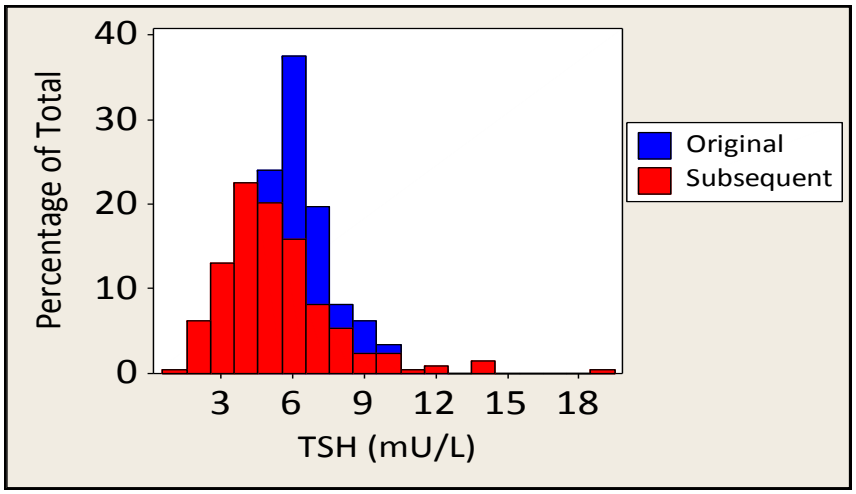


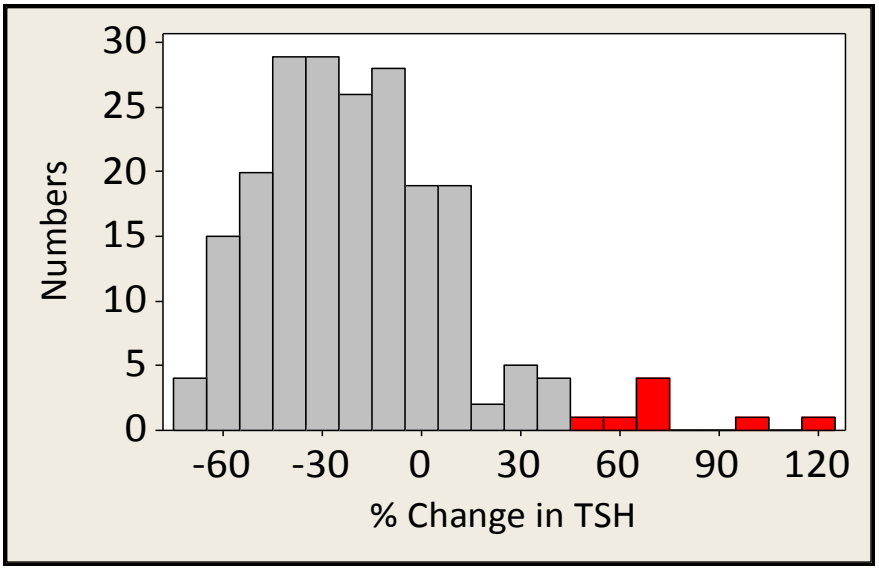
Figure 3.10: Superimposed histogram of original and subsequent TSHs



In figures 3.8-3.10 these data relate to those subjects who participated in the study (n=208).

I evaluated the "percentage (%) change in TSH" on interval testing across the cohort (figure 3.11). This identified a rise >40% from baseline in 5% of the cohort. Of these subjects half had a TSH <10mU/L and *could* represent a group at higher risk of hypothyroidism (see table 3.3).

Figure 3.11: Histogram depicting percentage change in TSH on interval testing



Subjects with a >40% rise in TSH are represented by red bars, all others are grey.

Table 3.3: Characteristics and thyroid parameters of five subjects with a ≥40% increase in TSH but a subsequent TSH <10mU/L.

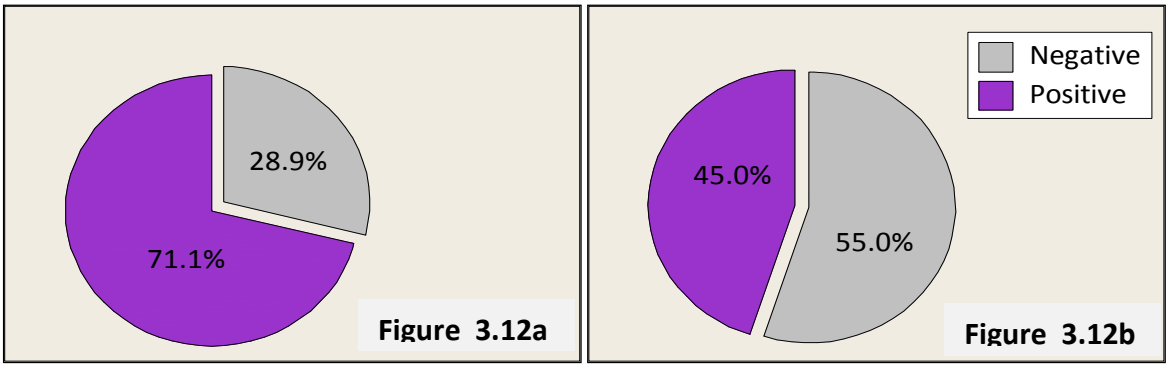
Study Number	Sex	Age (years)	TPO Status	Subsequent TSH (mU/L)	% change in TSH	Subsequent T4 (pmol/L)	% change in T4
180	F	45	+ve	7.6	+40	14.3	-6
181	F	61	+ve	9.3	+40	11.9	0
137	F	65	-ve	7.3	+41	13.0	+20
210	F	68	+ve	8.9	+66	12.2	-3
87	F	46	+ve	9.1	+70	12.1	-15

In this table F is for female, +ve and -ve indicates their TPO antibody status. Percentage (%) change in T4 and TSH are described as a rise (+) or fall (-) in value relative to original values. T3 was normal in all cases.

TSH changed appreciably (which I define as a >5% change) rising in 18% (38/208) ~70% of whom were TPO +ve. This TSH rise was associated with an appreciable fall in free-T4 (>5% drop) in ten subjects (25%).

When I compare those with an *appreciable* change in TSH on interval testing (rise versus a fall (figure 3.12); a greater proportion with TPO positivity were in the TSH rise group (71% versus 45%; 95% C.I. for the difference +10 to +42%, p-value 0.002). Ethnicities, sex ratios and % change in T4 were similar across both groups.

Figure 3.12: Comparing the TPO positive to negative ratio in subjects with a >5% rise in TSH values (3.12a) versus those with a >5% drop in TSH values (3.12b)

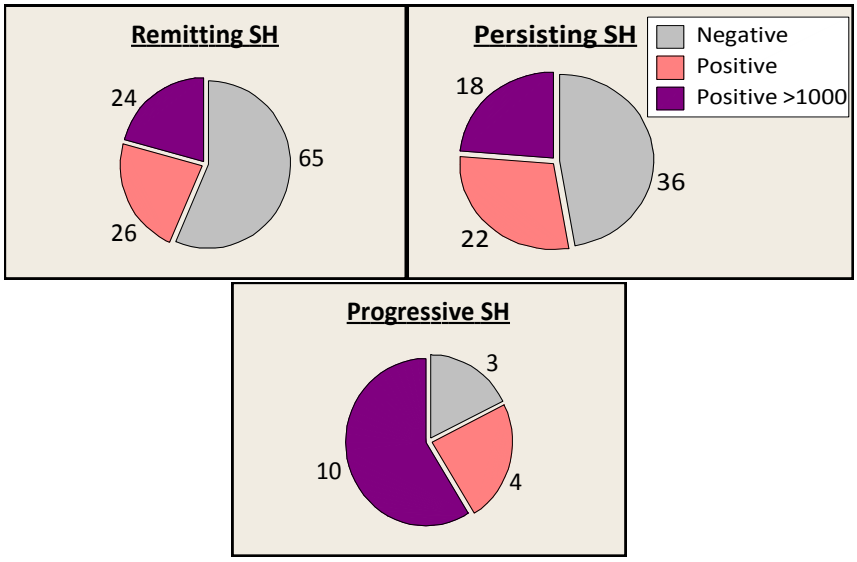


The subject numbers per group were 38/208 in the >5% rise in TSH group (3.12a) and 151/208 in the >5% fall in TSH group (3.12b). The legend is as shown.

When I evaluated the TPO antibody titre (categorised as negative (<6mU/L), positive (6-999mU/L) or strongly positive (>1000mU/L) according to SH natural history; remitting SH (TSH: <5mU/L), persisting SH (TSH: 5 to <10mU/L) or progressive SH (TSH: ≥10mU/L or TSH rise of >40%). It was evident that TPO antibody titre associated with progression of SH (figure 3.13).

I fund that there was a substantially smaller proportion with negative antibodies in the remitting (57%) versus the progressive SH group (18%) (p-value for the difference; <0.001), and a higher proportion with strongly positive antibodies in the progressive (60%) versus remitting (21%) or persisting groups (24%) (p-value for the difference; 0.002 and 0.006 respectively).

Figure 3.13: Displays the natural history of SH on repeat TFTs in relation to TPO antibody titre.



The numbers of subjects in each subcategory are provided. Study participation TSHs were <5mU/L in the remitting category; 5 to 9.9mU/L in the persisting category and ≥10 or a >40% rise from baseline TSH in the progressive SH category.

3.4.2 Evaluating the subgroup with a TSH ≥10 mU/L

This group were evaluated separately as a TSH ≥10mU/L represents the threshold at which thyroxine initiation is generally recommended (ATA/AACE guidelines Garber *et al* 2012). Of these 12 subjects (representing 6% of the cohort); 10 (83%) were TPO +ve, half described a FH of thyroid dysfunction, and their ages ranged from 20 to 64 years (see table 3.4). Other observations were as follows:-

- A TSH ≥10mU/L was identified on original TFTs in 5 subjects, 2.5%, and on participation TFTs in 10 subjects, 5% of the total cohort.

- Only 2 subjects amongst this group demonstrated a fall in TSH on re-testing; both male, 1 was TPO +ve (TSH; 10-7.3mU/L), and one TPO -ve (TSH; 11.46-5.53mU/L). Therefore a TSH ≥10 does not preclude a fall in TSH.

- T4 fell significantly (>5%) in 3 of the 12 subjects and below the normal reference range (<9pmol/L) in one subject (Number 093; T4 8.7pmol/L). T3 was normal in all cases.

- This group was similar to the total cohort in regard to:-

  - mean % change in T4 (figure 3.14 versus figures 3.15 and 3.16)

  -The proportion with a FH of thyroid dysfunction

  -Sex ratio (despite a greater proportion of males in this group (42% (5/12) versus 25% in the total cohort; the difference was not statistically significant (p-value 0.25)).

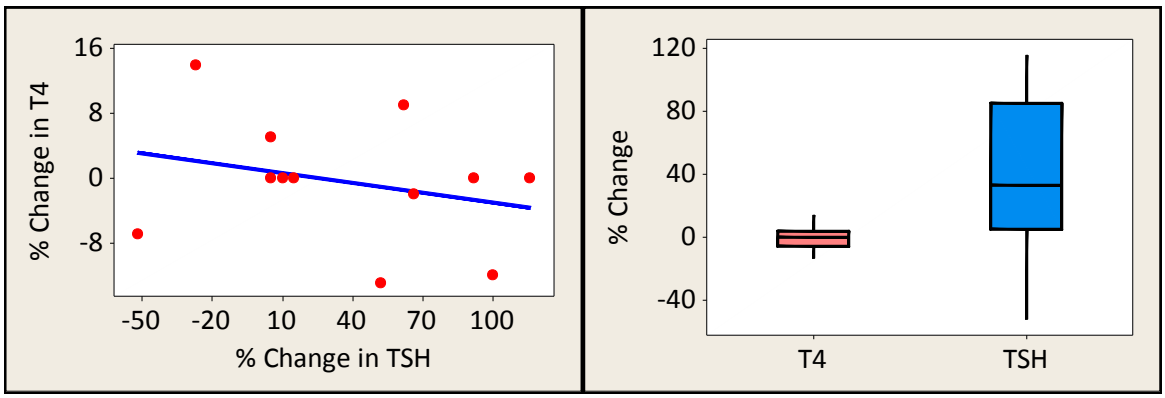
- This group was dissimilar to the total cohort in regard to:-

  - mean % change in TSH (+35 (≥10mU/L) versus -22 (Difference; +57%, p-value 0.003)

  - TPO positivity (75% (≥10mU/L) versus 50% (Difference; 25%, p-value 0.04)

Figure 3.14: Percentage change in T4 and TSH on interval testing in subjects with a TSH ≥10mU/L

Figure 3.14a: Scattergraph data **Figure 3.14b: Boxplot data**



In Figure 3.14a the red dots represent subject values and the blue line is the best linear relationship between these parameters. In Figure 3.14b the boxplots display the median values and the 25th and 75th quartile points. The whiskers indicate the range of values.

Table 3.4: TFTs and basic characteristics of subjects with a TSH ≥10mU/L

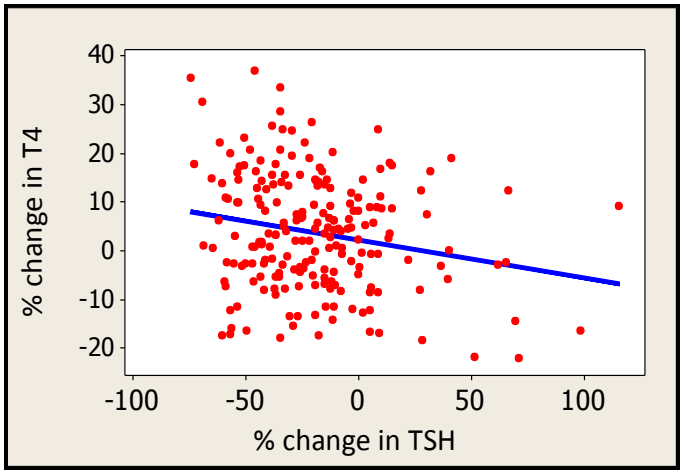
Study No	Sex	Age (Years)	Original TSH (mU/L)	Study TSH (mU/L)	% Δ TSH	Initial T4 (pmol/L)	StudyT4 (pmol/L)	% Δ T4	TPO Status	TPO titre <6; >1000	FH
103	F	20	6.29	10.19	+62%	12.91	14.1	+9%	+ve	>1000	No
164	M	62	6.56	14.15	+116%	10.7	10.7	0	+ve	625	Yes
132	M	43	6.85	10.4	+52%	10.32	9	-13%	+ve	>1000	Yes; cancer
93	F	45	7.08	14.06	+100%	9.87	8.7	-12%	-ve	1.9	No
90	F	32	8.19	13.63	+66%	12.59	12.3	-2%	+ve	>1000	Yes
198	F	32	9.21	10.1	+10%	11	11	0	+ve	689	Yes
200	M	51	9.73	18.68	+92%	9.2	9.2	0	+ve	682	No
4	M	31	10	7.31	-27%	12.87	14.7	+14%	+ve	>1000	No
174	F	63	10.02	10.54	+5%	13.4	13.4	0	-ve	6	No
51	F	67	10.3	11.83	+15%	13.03	13	0	+ve	>1000	No
18	M	68	11.46	5.53	-52%	13.93	13	-7%	-ve	1.1	Yes
108	F	44	11.76	12.39	+5%	10.82	11.3	+5%	+ve	886	Yes

The abbreviations in this table are No (for Number), F for Female and M for Male, Δ is the symbol for 'change', + and - indicate positive and negative directions of change respectively, +ve and -ve indicate TPO positivity and negativity respectively. FH for Thyroid Family history. Highlighted in red are; Females, TSH readings ≥10 mU/l, a fall in T4, positive TPO antibodies and a positive family history (FH) of thyroid disorders.

3.4.3 Exploring the interval change in TSH versus T4 across the cohort

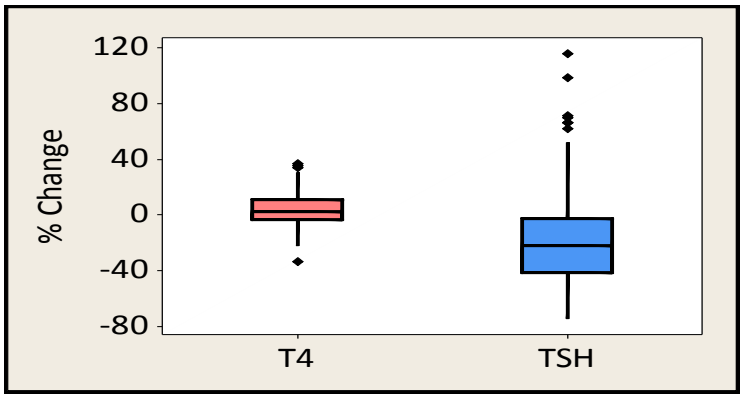
This was evaluated on the expectation that an inverse correlation between percentage change in T4 and TSH would be revealed (Hoermann *et al* 2010). A small, negative relationship between these parameters was seen (correlation coefficient (R) -0.07, p-value 0.02). In general a small change in T4 was associated with a much larger change in TSH consistent with the inverse log linear relationship described between these parameters (Hoermann *et al 2010*), (See figures 3.15 and 3.16 below).

Figure 3.15: Percentage change in TSH versus T4 on interval testing



The percentage (%) change in individual's free-T4 and TSH values are shown by red spots. The blue line represents the best linear relatonship between these parameters.

Figure 3.16: Boxplot illustrating the percentage change in T4 and TSH on interval testing

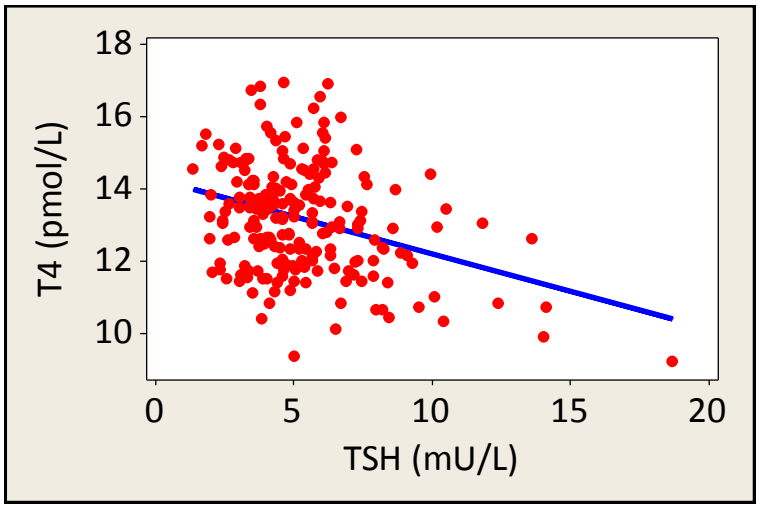


The percentage (%) change in value is shown on the y-axis. The box plots display the median value together with the 25th and 75th percentiles. Whiskers represent the upper and lower 25% distribution (excluding outliers). Outliers are represented by diamond shaped dots.

3.4.4 Evaluating TFT-component associations across the cohort

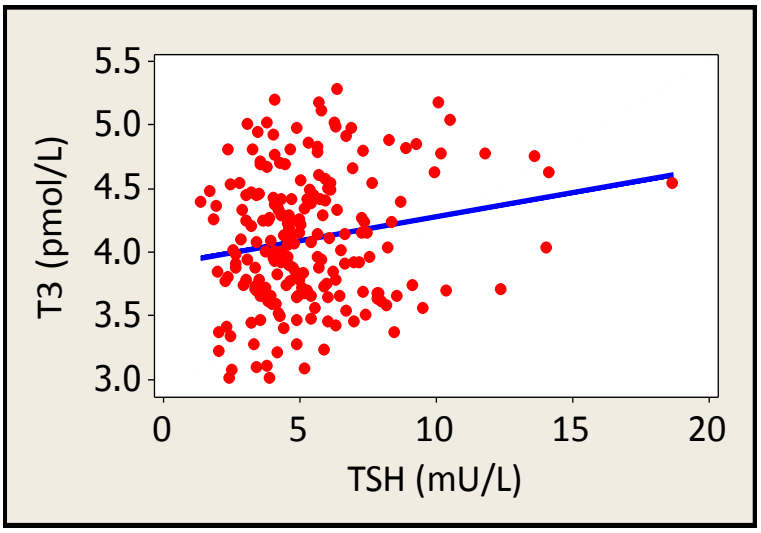
In this section the TFT components taken at study participation are evaluated across the cohort. This revealed a negative relationship between TSH and T4 (figure 3.17), an unexpected positive association between TSH and T3 (figure 3.18), and a positive association between T4 and T3 (figure 3.19). The regression equations are provided below each figure.

Figure 3.17: Relationship between TSH and T4 across the cohort



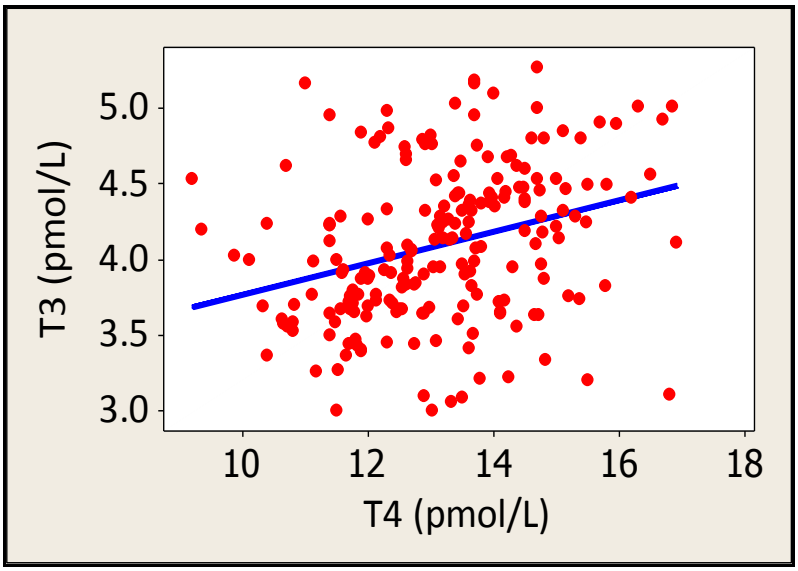
Regression equation; T4 = 14.2 - 0.21 x TSH, p-value <0.001, R^2 11%

Figure 3.18: Relationship between TSH and T3 across the cohort



Regression equation; T3= 3.9 + 0.04 x TSH, p-value 0.01, R^2 3.3%

Figure 3.19: Relationship between T4 and T3 across the cohort



Regression equation; T3 = 2.7 + 0.1 T4, p-value <0.001, R^2 10%

In figures 3.17-3.19 the red spots represent individual subject values and the blue line represents the best linear relationship between these parameters.

3.5 Genetic analyses on the thyrotrophin receptor

This was determined by dHPLC screening all 10 exons of the TSHR, on all study participants. When sequence aberration was suggested, direct DNA sequencing was undertaken to determine and confirm the sequence variant. The methods are all described in chapter 2.

3.5.1 TSHR mutations

Twelve TSHR-Ms (or suspected mutations) in twelve study subjects were identified. Table 3.5 details the basic thyroid biochemistry and characteristics of these subjects.

The salient observations on this group were as follows:-

- All but one subject had unmeasurable TPO antibodies. The exception (subject 022) had low-positive antibodies (titre 61 KU/L; reference range <6 to >1000). This observation is substantially different from the total cohort data where half were TPO antibody positive (difference of ~-42%, p-value <0.001)

- The TSH on testing ranged from ~2.9 to 9.1mU/L with a mean of 5.7 and a median of 5.9mU/L (the TFTS obtained on the screening and study participation samples were included in generating these figures).
- 5 of 12 were male (42%) representing a higher male proportion than in the total cohort (25%). However the difference did not meet statistical significance (p-value 0.3)
- 1 in 12 (~8%) were Asian; similar to the total cohort data (~9% Asian).
- 5 of 12 (42%) described a FH of thyroid disorders (a similar proportion to total cohort).
- Age was more evenly distributed across the recruitment ages whereas total cohort data was skewed towards older ages. The mean ages were similar.
- The W546X mutation was identified in 7 of 12 subjects (3% of the total cohort) indicating a prevalence 6x higher than the assumed background rate of ~1/180(Jordan *et al* 2003).
- 10 of the 12 mutations resided in exon 10. The two exceptions were the P162A in exon 6, and the Y195C in exon 7.
- The R531W (Exon 10) and the P162A (Exon 6) identified are both known mutations (Cangul *et al* 2010 & Sunthornthepvarakul *et al* 1995).

Two novel sequence variants were identified:-

- The W488X (identified in subjects 022 and 111); where a sequence terminating amino acid (X) replaces tryptophan (W) at codon 488 (Exon 10). The identically positioned W488R is a recognised inactivating TSHR-M (De Marco *et al* 2009).
- The Y195C (subject 032) is a sequence variant where Cysteine (C) replaces tyrosine (Y) at codon 195 (exon 7). Both amino acids are polar and therefore the functional significance of this substitution needs to be determined.

Although the functional impact of the Y195C on TSHR signalling needs to be determined; for the purpose of my analyses this group of 12 subjects are considered 'The mutation group'.

Table 3.5: Basic characteristics and thyroid biochemistry in the TSHR mutation group.

Study Number	Sex, Age & Race	Sequence variance & exon	Original TSH (mU/L)	Study TSH (mU/L)	Study T4 (pmol/L)	Study T3 (pmol/L)	TPO titre (<6; >1000) (KU/L)	Thyroid Family History
003	F, 34, C	W546X 10	7.88	3.4	12.9	3.09	<6	Yes
022	F, 40, C	W488X 10	6.11	4.88	13.65	3.82	61	No
032	M, 43, A	Y195C 7	6.45	3.42	13.72	4.5	<6	No
046	F, 61, C	R531W 10	5.8	7.66	14.07	4.53	<6	No
078	M, 38, C	P162A 6	5.69	6.16	15.37	3.74	<6	No
084	F, 44, C	W546X 10	5.28	4.65	11.72	3.76	<6	No
099	M, 63, C	W546X 10	9.1	5.95	13.62	4.39	<6	No
111	F, 66, C	W488X 10	6.45	5.66	13.04	3.95	<6	Yes
119	F, 52, C	W546X 10	6.17	4.03	12.74	3.83	<6	Yes
148	M, 38, C	W546X 10	6.98	7.01	11.7	3.44	<6	Yes
172	M, 61, C	W546X 10	6.6	4.18	15.5	2.87	<6	No
177	F, 58, C	W546X 10	5.46	2.88	15.1	4.32	<6	Yes

Sex is categorised as female (F) or male (M). Age is recorded in years, race is categorised as Asian (A) or Caucasian (C). The family history was considered positive when hyperthyroidism, hypothyroidism or SH affected a first or second degree relative.

3.5.2 D727E polymorphism group

Nine subjects (4% of the cohort) were identified as being heterozygous carriers of the D727E polymorphism. This is substantially less than the 10% prevalence reported in a British and Canadian control cohort (Matakidou *et al* 2004). Table 3.6 details their thyroid biochemistry and basic characteristics.

Table 3.6: Basic characteristics and thyroid biochemistry in the D727E polymorphism group

Study Number	Sex, Age & Race	Original TSH (mU/L)	Study TSH (mU/L)	Study T4 (pmol/L)	Study T3 (pmol/L)	TPO (KU/L)	Family History
012	F, 38, C	6.14	6.68	11.8	3.65	<6	Yes
081	F, 63, C	6.31	4.75	11.88	3.87	<6	Yes
096	M, 39, A	9.03	7.16	11.58	3.91	<6	No
124	F, 56, C	6.06	4.6	11.57	4.28	6	No
132	M, 43, C	6.85	10.4	10.32	3.69	>1000	Yes
158	F, 31, C	5.28	3.26	14.8	4.8	<6	Yes
184	F, 47, C	5.6	3.54	12.6	4.7	<6	Yes
190	F, 46, C	8.36	4.58	15	4.22	<6	Yes
193	F, 59, C	5.54	6.38	14.7	5.27	<6	No

Sex is categorised as female (F) or male (M). Age is recorded in years, and race categorised as Asian (A) or Caucasian (C). The family history was positive if hyper or hypothyroidism/SH was reported in a first or second degree relative.

The salient observations on the D727E group were as follows:-

- Eight of nine had negative TPO autoantibodies; substantially different from the total cohort data (difference minus ~40%, p-value <0.001).
- The male to female ratio (2: 7) was similar to the total cohort, as was the proportion reporting a thyroid FH (66% versus ~40% of in total cohort data, p-value 0.1)

3.5.3 The P52T polymorphism group

Thirteen subjects carrying the P52T polymorphism were identified giving a prevalence of ~6%, similar to that reported amongst control populations (Simanainen *et al* 1999). Table 3.7 details their thyroid biochemistry and basic characteristics. The salient observations on this group were as follows:-

- There was a higher proportion of Asian subjects (6/13) in this group than in the total cohort (difference ~ +40%, p-value 0.008). This potentially reflects different gene pools in subjects of different ethnic origins.
- Five of thirteen had positive thyroid autoantibodies, a ratio similar to the total cohort (difference minus ~10%, p-value 0.4).
- The female to male ratio (11:2), was similar to the total cohort (difference ~+10%, p-value 0.4).
- Four of thirteen reported a FH of thyroid disease, similar to the total cohort (difference minus 10%, p-value 0.5).

3.5.4 The D36H polymorphism

This polymorphism was identified in one subject suggesting a prevalence similar to the 0.5% prevalence described in control cohorts (Peeters *et al* 2003). This subject was a 69 year old Caucasian female who was TPO antibody negative and her TSH normalised at study attendance (5.8 to 1.8mU/L).

Table 3.7: Characteristics of the P52T polymorphism group.

Study Number	Sex and Race	Screening TSH (mU/L)	Study TSH (mU/L)	Study T4 (pmol/L)	Study T3 (pmol/L)	TPO antibodies (KU/L)	Thyroid Family History
25	F, 59, C	5.92	4.27	14.29	4.7	>1300	No
26	F, 37, A	6.5	3.51	14.2	4.45	>1300	No
42	F,59, C	9.48	8.47	10.4	3.36	>1300	Yes
92	F, 51, A	3.79	5.39	11.75	3.79	<6	No
97	M, 64, A	3.92	6.12	13.63	3.92	<6	No
104	F, 54, C	3.43	7.52	11.83	3.43	<6	No
110	M, 67, A	4.32	5.64	12.9	4.32	<6	No
130	F, 56, A	4.26	6.29	13.3	4.26	30	Yes
154	F, 47, A	3.72	5.71	14.1	3.72	<6	No
184	F, 47, C	4.7	5.6	12.6	4.7	<6	Yes
195	F, 64, C	5	5.26	14.7	5	30	Yes
196	F, 58, C	3.1	5.66	16.8	3.1	<6	No
201	F, 49, C	3.64	6.3	14.1	4.53	<6	No

Sex is categorised as female (F) or male (M). Age is recorded in years, and race is categorised as Asian (A) or Caucasian (C). The family history was positive if hyper or hypothyroidism/SH was reported in a first or second degree relative.

3.6 TFT evaluations in the TSHR mutation & polymorphism groups versus the total cohort

The TFTs in the TSHR-M and D727E groups were compared (except for D727E subject 132, with ATD). All analyses here on relate to the TFTs taken at the time of study participation.

Although the likelihood of revealing true differences is limited by low subjects numbers; a lower T3 in the TSHR-M subjects (of borderline significance) is revealed (see table 3.8).

Table 3.8: Compares TFT values between the TSHR mutation and D727E carriers

TFT Parameter	$\bar{x}$ Mutation (n = 12)	$\bar{x}$ D727E (n = 8)	$\bar{x}$ Difference	95% C.I	p-value
T4 (pmol/L)	13.59	13	+0.6	-0.84 to 2.05	0.38
TSH (mU/L)	5.04	5.07	-0.03	-1.4 to 1.4	0.97
T3 (pmol/L)	3.8	4.34	-0.5	0.01 to -1	0.055

In this table; $\bar{x}$ represents the mean value, C.I represents the confidence interval and the plus (+) and minus (-) signs indicate the direction of the difference.

I subsequently evaluated the TFTs in the TSHR-Ms versus the rest of the *TPO negative* subjects (with study TSHs in a similar range; 2.8 to 9.1mU/L (excluding the D727E subjects)). This analysis revealed a lower mean T3 value in the TSHR-M group (Table 3.9).

Table 3.9: Compares TFT parameters between the mutation group and the TPO negative cohort across a similar TSH range.

TFT Parameter	Mutation $\bar{x}$ (n = 12)	TPO -ve cohort $\bar{x}$ (n=75)	$\bar{x}$ Difference	95% C.I	p-value
T4 (pmol/L)	13.59	13.67	-0.07	-0.95 to +0.8	0.86
TSH (mU/L)	5.04	4.8	+0.25	-0.72 to +1.2	0.59
T3 (pmol/L)	3.8	4.2	**-0.35**	-0.65 to -0.05	**0.026**

In this table: $\bar{x}$ represents the mean, C.I represents the confidence interval and the plus (+) and minus (-) signs indicate the direction of the difference.

I subsequently evaluated a range of parameters from the total cohort data that might influence T3 (including TSHR-M status). This was evaluated in a multiple regression analysis (MRA) as shown overleaf.

Please see sections 2.19, and 4.4.1 for an explanation of the use and interpretation of MRAs.

The parameters assessed against T3 in this MRA were TSHR-M, Male Sex, TSH, T4 and TPO antibody positivity. Table 3.10 details the results of this analysis.

$$\mathbf{T3} = 1.9 - 0.36\ \mathbf{TSHR\text{-}M} + 0.14\ \mathbf{Male(1)} + 0.76\ (\mathrm{Log}^{10}\mathbf{TSH}) + 0.13\ \mathbf{T4} - 0.04\ \mathbf{(TPO{+}ve)}$$

$$N=207, \qquad R^2 = 21\%$$

Table 3.10: MRA evaluating T3 against the predictors listed including TSHR-M status

Predictor	R	SE	95% C.I.	p-value
Constant	+1.9	0.36	+1.2 to +2.6	<0.001
TSHR-M	-0.36	0.14	-0.6 to -0.1	0.01
Male(1)	+0.14	0.08	-0.02 to +0.3	0.06
Log^{10}TSH (mU/L)	+0.76	0.19	+0.4 to +1.1	<0.001
T4 (pmol/L)	+0.13	0.023	+0.08 to +0.2	<0.001
TPO+ve(1)	-0.04	0.07	-0.18 to +0.1	0.6

In Table 3.10: The regression coefficient (R), standard error (SE) and confidence intervals (C.I) are provided. The plus (+) and minus (-) signs indicate the direction of the association.

The T3 MRA supports the observation that TSHR-M status correlates *negatively* with T3. This association was subsequently explored in a stepwise MRA (Model 3.1) which reveals strengthening of the relationship between T3 and mutation status as confounders are added in to the analysis; supporting an independent negative association between T3 and TSHR-M status.

Model 3.1: Stepwise regression model exploring the association between mutation-carrier status and T3; assessed singularly and subsequently adjusted for confounders.

N	R	95% C.I.	p-value
Step 1; crude (mutation carrier status against T3 only)			
207	-0.26	(-0.6, +0.03)	0.08
Step 2; Step 1 also adjusted for T4			
207	-0.31	(-0.6, -0.04)	0.03
Step 3; Step 2 also adjusted for log^{10}TSH			
207	-0.33	(-0.6, -0.06)	0.02
Step 4; Step 3 also adjusted for TPO antibody status			
207	-0.35	(-0.6, -0.08)	0.01
Step 5; Step 4 also adjusted for Male Sex			
207	-0.36	(-0.6, -0.09)	0.01

Model 3.1 details the number of subjects in the analysis (N), the regression coefficient (R), the 95% confidence interval (C.I) for each analysis step and its p-value. The minus (-) or plus signs (+) indicate the direction of the association.

I subsequently repeated the T3 MRA of table 3.10, but substited D727E status (excluding the subject with ATD) for TSHR-M status. This analysis did not reveal a clear association between T3 and D727E status (see table 3.11 where this MRA is summarised).

Table 3.11: MRA evaluating T3 against the predictors listed that include D727E status

Predictor	R	SE Coefficient	95% C.I.	p-value
Constant	+1.87	0.35	1.2 to 2.6	<0.001
D727E	+0.3	0.17	-0.03 to +0.6	0.08
Male Sex	+0.16	0.08	+0.02 to +0.34	0.05
Log^{10}TSH (mU/L)	+0.74	0.19	+0.1 to +0.37	<0.001
T4 (pmol/L)	+0.13	0.02	+0.1 to +0.17	<0.001

In Table 3.11: The Regression Coefficient (R), Standard Error (SE) and Confidence Intervals (C.I) are provided. The plus (+) and minus (-) signs indicate the direction of the association.

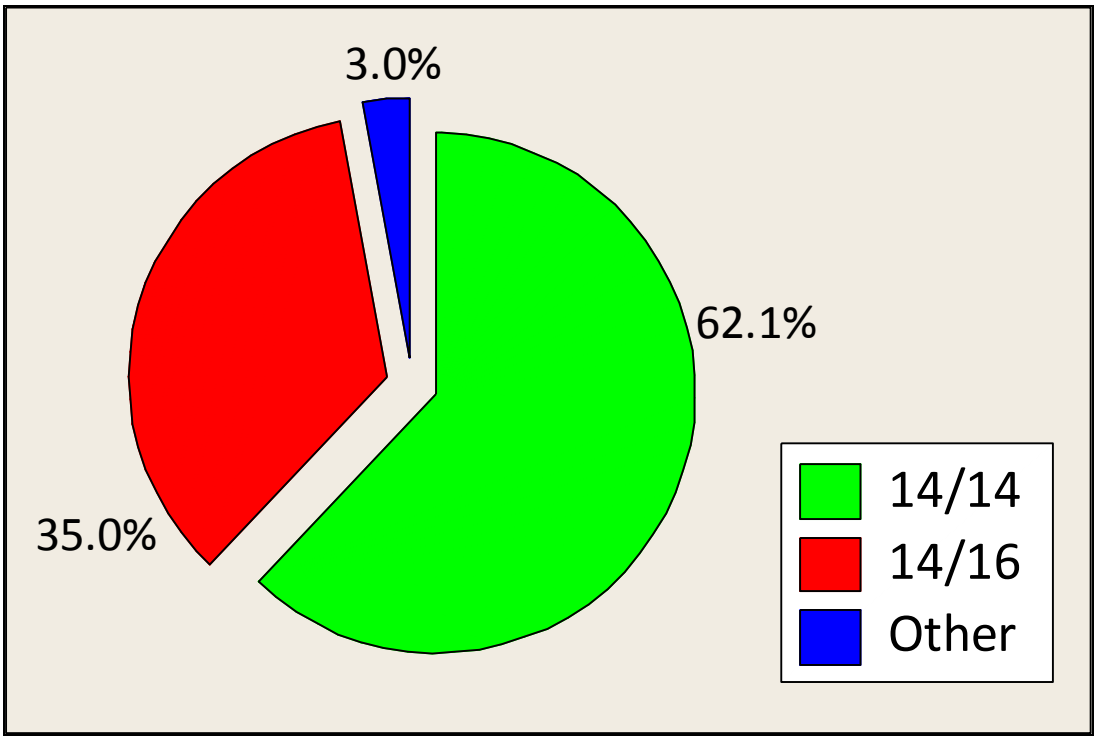
An equivalent MRA was performed for the P52T polymorphism group (n=13). This did not reveal an association between the P52T polymorphism and T3 values (R minus 0.06, p-value 0.55 (MRA not shown)).

3.7 FOXE1 polyalanine tract length polymorphism evaluations

FOXE1 polyalanine tract length (PTL) polymorphism status was determined in 203 study subjects (5 had insufficient quantity or quality of DNA for analysis).

This work revealed the homozygous FOXE1 14 PTL as the prevalent genotype (identified in 62% (126 subjects)), followed by the heterozygous 14/16 combination in 35% (71 subjects). Other combinations occurred in 3% (figure 3.20).

Figure 3.20: Pie chart depicting FOXE1 PTL genotype frequencies across the cohort



The FOXE1 PTL polymorphism genotype categories are indicated by the legend (homozygous$^{14/14}$ in green, heterozygous$^{14/16}$ in red, and all other combinations in blue).

3.7.1 Characteristics of the cohort according to FOXE1 PTL polymorphism status

The FOXE1 PTL polymorphism status across the cohort was evaluated as the 14/14 combination versus *all* others (see table 3.12). This revealed no differences between the groups in regard to;

- TPO antibody status
- Sex ratios
- Ethnicity
- T4 values
- TSH values

However a higher T3 amongst the FOXE1 $PTL^{14/14}$ group was revealed. This relationship was subsequently explored in a parsimonious stepwise MRA (Model 3.2) that supports this observation.

Table 3.12: Comparison between the $FOXE1^{14/14}$ PTL versus *all other* PTL combinations for the parameters listed.

Parameter	$\bar{x}$ 14/14 (n=126)	$\bar{x}$ Other (n=77)	$\bar{x}$ Difference	p-value
% TPO +ve	50	53	-3	0.61
% Male	24	26	-2	0.75
% Asian	9%	9%	<0.02	0.7
TSH (mU/L)	5.43	5.22	+0.21	0.52
T4 (pmol/L)	13.08	13.16	-0.08	0.72
T3 (pmol/L)	4.16	4.0	**+0.16**	**0.02**

In table 3.12: $\bar{x}$ represents the mean value in each group. The differences in the % between groups for each parameter listed, with respective p-values are provided.

Model 3.2: Stepwise regression model exploring the association between the FOXE1$^{14/14}$ PTL status and T3 values; assessed singularly and subsequently adjusted for confounders.

N	R	95% C.I.	p-value
Step 1; crude (14/14 status against T3 only)			
202	+0.17	(+0.03, +0.3)	0.02
Step 2; Step 1 also adjusted for T4			
202	+0.18	(+0.04, +0.32)	0.009
Step 3; Step 2 also adjusted for log^{10}TSH			
202	+0.18	(+0.05, +0.3)	0.008
Step 4; Step 3 also adjusted for TSHR mutation status			
202	+0.2	(+0.06, +0.32)	0.005

Model 3.2 details the number of subjects in the analysis (N), the regression coefficient (R), the 95% confidence interval (C.I) at each step and its respective p-value. The minus (-) or plus signs (+) indicate the direction of the association.

3.7.2 FOXE1 PTL status in association with TSHR gene sequence variance

FOXE1 PTL polymorphism status could be assessed in 11 of 12 TSHR-M subjects and 8 of 9 D727E subjects. Basic analysis revealed a lower than expected prevalence of the FOXE1$^{14/14}$ PTL polymorphism genotype amongst the D727E subjects, but no differences from total cohort data amongst the TSHR-M group (see table 3.13).

Table 3.13: Comparison of FOXE1$^{14/14}$ genotype frequencies between the TSHR-M or D727E groups versus total cohort data

FOXE1$^{14/14}$ genotype frequency in TSHR group	FOXE1$^{14/14}$ genotype frequency in TC (n=203)	$\bar{x}$ Difference	95% C.I	p-value
Mutation (n=11); 0.73	0.62	+0.11	-0.16, +0.4	0.4
D727E (n=8); 0.25	0.62	-0.4	-0.12, -0.7	0.005

In this table; $\bar{x}$ stands for mean, TC for Total Cohort and C.I for Confidence Interval.

3.8 Discussion

3.8.1 Recruitment

Study recruitment fell short of anticipated numbers with 208 recruited rather than the target number of 350. The study protocol accurately predicted the number of subjects to be offered study participation (463 versus 500 expected). However, a recruitment rate of 45% fell short of the 70% anticipated. The expectation that 30% would *not* be recruited was similar to the 34% of subjects 'unwilling' or uncontactable. However, the fact that ~20% of GP approved subjects were unsuitable for the study and that only 45% of GPs would respond to our letter of approach was not expected (figures 3.2 and 3.3 respectively).

The recruitment rates achieved were similar to those reported by Cornelia *et al* (2005) who quoted 30-50%. Of the patients I approached directly, 65% wished to participate although 15% were unsuitable. Thereafter dropout rates were low, with ~5% (12/220) failing to attend study appointments, and 3% (6/208) failing to attend DXA appointments. Dropout rates were considerably less than the >20% rate reported by Bell *et al* 2013. High attendance rates may reflect my practice of re-contacting and rearranging appointments for subjects who failed to attend. In this study a subject's sex did not influence their tendency to participate (Table 3.1).

GP participation rates of 45% were considerably less than those reported in other studies (~80% Roland *et al* 2001). The reason for this is unclear but presumably reflects GP's disinclination locally to take on this additional workload.

3.8.2 Demographics

A female preponderance (75%) and an age distribution favouring older subjects (modal age 65 years (see Figure 3.5) was revealed. This reflects the fact that SH (like primary hypothyroidism) is more prevalent in women and with advancing age (Canaris *et al* 2000, Hollowell *et al* 2002). The Whickham Study reported SH in 7.5% of women and 2.8% of men (Tunbridge *et al* 1977); a similar sex ratio was observed in this study. The antibody status of the cohort was 50% positive and 50% negative, an observation falling within the reported range for areas traditionally considerd to be iodine sufficient (~40% (Surks & Hollowell 2007) to 80% (Allan *et al* 2000) but a lower prevalence of TPO positivity than anticipated in the study

protocol (Appendix 1 & section 2.1.3; 2/3^{rds} TPO +ve were anticipated). The ethnicity of participants (90% Caucasian, 9% Asian and 1% of Afro-Caribbean) suggested that Asian subjects may be slightly over represented in this study (2011 census data reported; 90% Caucasian, 5% Asian, 1% Black and 3% 'other ethnicities' (census data from the Cardiff and Vale Health Board, table LC2101EW; Ethnic group by Sex and Age) reflecting my observation that GPs working in areas where ethnic minorities are prevalent in Cardiff (ie; Grangetown, Cardiff Bay and Riverside) were active referrers to the study.

The comorbidity rates amongst study participants appeared to be higher than that described for the local population (Director of public health's report for Cardiff and Vale Health Board 2012); Hypertension affected 30% versus 10% of the local population; Ischaemic Heart Disease affected 5% versus 2.5% of the local population and 68% of participants were overweight (BMI >25) versus 53% of the local population. However, the fact that these figures are not age group and sex ratio equivalent limits their utility in drawing inferences about the recruited cohort. Nevertheless it is possible that the SH cohort may have poorer health status than the local population although individuals with poorer health status might be more likely to be recruited to the study.

The self-reported lifestyle behaviour amongst participants was similar to that described for the local population (Director of public health's report for Cardiff and Vale Health Board 2012); 30% had a significant smoking history and 13% were current smokers (versus 21% of the local population), 50% described their lifestyle as sedentary (whereas 75% of the local population are known *not* to meet recommended activity levels). Only 7% confessed to drinking above weekly recommended alcohol limits (versus 45% of the local population) suggesting that self-reported alcohol intake *may* have been underestimated.

3.8.3 Thyroid function tests

The TFTs at study participation revealed TSH to have fallen in ~80% of the cohort (160/208), falling back into the reference range in ~half (figure 3.9). This scenario is recognised with SH remittance rates of 25% (Parle *et al* 1991) to 50% (Diez and Iglesias 2009) described.

The TSH rose in ~20% (43/208) of the cohort with ~6% (12/208) meeting standard criteria for TRT (TSH ≥10mU/L; Surks *et al* 2004). A rise ≥40% from baseline was apparent in ~5% of the

cohort (figure 3.11) and I postulate that this group might represent subjects with more rapidly progressive thyroid dysfunction who may also benefit from TRT (if so increasing numbers requiring TRT to 17 subjects (~8%)). However, this is not described.

The Whickham Study reported progression from SH to PH to affect ~ 4.3% per year amongst TPO +ve females, versus ~2.6% per year amongst TPO -ve females (Tunbridge *et al* 1977). In my cohort progression of SH (to a ≥10mU/L) within the ~3 month TFT re-evaluation period occurred in ~10% of the TPO +ve group (10/104) and 2% (2/104) of the TPO -ve group. Thus supporting the notion that progression of SH is more common amongst TPO +ve subjects.

Twenty year follow-up of The Whickham Study reported progression of SH as ~4x higher amongst TPO antibody +ve men with SH than women (Vanderpump *et al* 1995). I too observe greater progression (to TSH ~≥10) amongst TPO +ve men (4/17; 23%) versus women (6/87; 7%) although this difference did not meet statistical significance (p-value 0.12).

Progression of SH correlated with TPO antibody titre in this cohort (Figure 3.13) as also reported by Zhang *et al* 2010. However half of the subjects with progressive SH (to a TSH ~≥10mU/L had a TPO titre <1000 (TPO titres; 163, 170, 689, 625, 689 mU/L).

Exploration of the relative change in T4 and TSH values across cohort (Figures 3.15 & 3.16); and in progressive SH (Figure 3.14) revealed a small negative association between these parameters. However, on an individual subject level change in T4 was a poor indicator of change in TSH. I found that free-T4 tended to remain relatively constant in both groups despite marked fluxes in TSH. This observation would potentially support the use of TSH-only assays to monitor well patients with diagnosed SH in the community setting.

The TFT associations across the cohort revealed an expected negative relationship between TSH and free-T4 (figure 3.17); a positive relationship between free-T4 and free-T3 (figure 3.19) and an unexpected positive association between TSH and free-T3 (figure 3.20). This suggests that there may be over compensation of the mechanism to maintain normal free-T3 levels in compensated SH (T3 is the active cellular hormone and is markedly more potent than T4 (Gross & Pitt-Rivers 1953)). The suggestion that progressive SH may be associated with increased thyroid hormone efficacy at the tissue level (rather than the inverse of this) would

be a new concept. However, the possibility that this small rise in T3 compensates for the more substantial fall in T4 remains.

A rising T3 in SH might be driven by peripheral effects (i.e. deiodinase enzyme mediated), central effects (i.e. modulating the T3:T4 synthetic ratio from the thyroid gland) and/ or modulation of T3 degradation and excretion. A falling T4 upregulates DIO2 to increase peripheral T3 synthesis however this might predict a negative relationship between T4 and T3 which is not seen. TSH might be driving deiodinase effects as reported in osteoblasts (Morimura *et al* 2005), skeletal muscle (Hosoi *et al* 1999) and thyroid tissue (Murakami *et al* 2001b). However, further investigation is required firstly to explore intra-subject TFT variability with progression of SH (for verification purposes), and secondly to elucidate its physiological mechanism.

3.8.4 TSHR mutations

Twelve TSHR sequence variants (SVs) in 12 subjects were identified. Eleven of whom were TPO antibody negative, and one had low-positive antibody titres. This supports the notion that TSHR-Ms lie overwhelmingly in seronegative SH cohorts (Alberti *et al* 2002). Their prevalence was 6% across the cohort and ~11% amongst seronegative subjects which is equivalent to the prevalence reported in other seronegative SH cohorts (Camilot *et al* 2005, Nicoletti *et al* 2009 and Tonacchera *et al* 2004).

Exon 10 is the largest of the TSHR exons being more than twice the size of the other 9 exons combined (Iosco & Rhoden 2009). The majority of TSHR-Ms reside here (~123 of 147 mutations and 32 of 51 inactivating mutations (Paschke 2013; TSH receptor database)). Eleven of the twelve SVs identified in this study reside in Exon 10. However, this was not the observation of the aforementioned Italian studies; Nicoletti *et al* (2009) identified 5 of their 11 mutations in Exon 10 (2 x Exon 1; 1 x Exon 2; 1 x Exon 4; 1 x Exon 5 and 1 x Exon 6), Camilot *et al* identified 4 of 13 mutations in Exon 10 (1 x Exon 9, 1 x Exon 6, 2 x Exon 4, and 5 x Exon 1), and Tonacchera's group did not report any of their 5 familial mutations to be in Exon 10 (3 x (P162A) Exon 6, and 2 x (L252P) Exon 9).

A predominance of SVs in exon 10 in my cohort is likely to reflect the local South Wales gene pool. Indeed the W546X mutation that renders the TSHR non-functional (Clifton-Bligh *et al*

1997) was 6x more prevalent in this cohort than a local control cohort in whom TFTs were unknown (Jordan *et al* 2003). The novel W488X mutation was identified in two study subjects. Observations on the SV group (table 3.5) suggested a modest female prevalence (7 Female: 5 Male) compared to the total cohort ratio (3:1). Exploring this further I found other studies where a male predominance for inactivating TSHR-Ms were described. Nicoletti *et al* (2005) described mutations in 7 SH males versus 3 SH females. A study from Taiwan evaluating the R450H TSHR mutation in Congenital Hypothyroidism (CH) reported 4 of 5 subjects to be male (despite a female excess within this population (Chang *et al* 2012)). In our unit male siblings homozygous for the TSHR mutation W546X were reported (Jordan *et al* 2003), and Cerbone *et al* (2013) report male siblings heterozygous for TSHR inactivating mutations. Given that a higher prevalence of TSHR-Ms would not be expected in males, it might be that their clinical effects are more pronounced. However, further study to verify and explore this suggestion are required.

The TSH ranged between 2.9 and 9.3 mU/L in the SV group. Given that subjects were recruited because their original TSH was ≥5 mU/L and subjects with TSH ≥10 mU/L were generally unsuitable for the study (as TRT commenced) this may not accurately reflect the TSH range in this subgroup. Previous studies suggest that the natural history of subjects with partial TSH resistance is *not* one of progressive thyroid dysfunction (Tenenbaum-Rakover *et al* 2009). Genotype-phenotype correlations in this group are poor, reflected by the fact that heterozygous mutations have been identified at neonatal screening (reflecting more extreme TSH levels (Tenenbaum-Rakover *et al* 2009, Camilot *et al* 2005) and also rarely identified with a normal TSH (<2mU/L) (Clifton-Bligh *et al* 1997).

TSHR inactivating mutations are generally considered to be transmitted in an autosomal recessive (AR) manner (de Roux *et al* 1996, Jordan *et al* 2003, Park *et al* 2004), presumably reflecting the fact that homozygous or compound heterozygous inactivating mutations are typically associated with CH. As such, we may have expected this group to infrequently report a family history (FH) of thyroid disease, however ~half did. However, *it is* recognised that heterozygous inactivating TSHR-Ms commonly run in families (Tonacchera *et al* 2004, Tenenbaum-Rakover *et al* 2009) suggesting an AR inheritance pattern may be misleading and a partial dominant inheritance pattern for SH may be more accurate. Indeed several studies

support this notion (Cerbone *et al* 2013, Calibiro *et al* 200, Alberti *et al* 2002). Interestingly, a thyroid FH was less frequently reported in the TPO negative group (~30%) with TSHR-Ms removed.

Of the SVs identified in the cohort; The R531W and the P162A are known mutations. Both have been reported in their homozygous state in neonates (identified at neonatal screening), each with normally positioned thyroid glands and normal T4s. TSHR modelling analysis suggested a mild destabilising effect from each mutation (Cangul *et al* 2010).

The W488X and the Y195C identified are novel SVs. W488X is a nonsense mutation that will truncate the receptor and is expected to impact on TSHR function. In the Y195C (exon 7) cysteine replaces tyrosine in the hinge region of the TSHR. Although both of these amino acids are polar, disulphide bonds between cysteine residues can be important to 3D modelling (Betz SF, 1993) and therefore this might impact on TSHR structure and function. However needs to be determined.

I evaluated TFT parameters in the SVs against the D727E subjects and the rest of the cohort (section 3.6). Interestingly this revealed lower T3 values (relative to other TFT parameters) in the TSHR-M group. This observation potentially fits in with thyroid hormone physiology given that TSHR activation on thyroid follicular cells triggers a cascade of events resulting in thyroid hormone synthesis (Vassart & Dumont 1992). As such partial TSHR resistance related to heterozygous inactivating mutations might be expected to impact on this process.
It is also possible that TSH (acting via the TSHR) moderates deiodinase enzyme activities peripherally (as suggested in brown fat and osteoblasts (Morimura *et al* 2005)) accounting for lower T3 values (relative to T4 and TSH) in these subjects.

3.8.5 D727E polymorphism

The D727E polymorphism was identified in ~4% of the cohort, a lower prevalence than described amongst control groups (10% (Matakidou *et al* 2004) to 16% (Mühlberg *et al* 2000). This may reflect its association with increased TSHR sensitivity (Gabriel *et al* 1999) and lower TSH values (Hansen *et al* 2007). It was therefore surprising to identify this in a group of predominantly TPO negative (8/9) subjects.

When I compared TFT associations in this group, a positive association with T3 was identified (Table 3.11) albeit not statistically significant. It is possible that this effect is being abrogated by a prevalence of the FOXE1$^{14/16}$ polymorphism in this group (negatively associated with T3). If the D727E polymorphism is positively associated with T3 then this would potentially support reports of enhanced TSHR sensitivity and lower TSH levels in this group. However, small subject numbers limits the reliability of my observations and postulations, as does the absence of euthyroid controls.

3.8.6 FOXE1 PTL polymorphisms

The FOXE1 PTL 14/14 polymorphism was the prevalent genotype in this cohort (62%), followed by the 14/16 (35%) (Figure 3.20). This is broadly similar to a Slovenian and New Zealand study that identified the 14/14 in 46% and the 14/16 in 36% of their controls (Watkins *et al* 2006), but differs from a Chinese study where 96% of controls (n = 106) had the 14/14 genotype and 4% had the 14/16 genotype.

A positive association between the 14/14 polymorphism and T3 levels (versus other PTL combinations) was revealed. Higher transcriptional activity in the 14 versus the 16 PTL polymorphism is described (Bullock *et al* 2012) which may be relevant to this observation. This observation is interesting and would suggest that common FOXE1 PTL polymorphisms are likely to influence TFTs.

The limitations to the study that are of relevance to this chapter are discussed in the concluding and closing chapter.

CHAPTER 4: BONE AND THRYOID FUNCTION PARAMETERS IN SUBCLINICAL HYPOTHYROIDISM

4.1 Introduction

Frank thyroid diseases are associated with changes in body composition. Changes in hypothyroidism include weight gain, myxoedematous tissues and organs (including muscle pseudohypertrophy (Foley 2004) and myopathies (Torres & Moxley 1990), and delayed linear growth and physical maturation in children. Changes of hyperthyroidism include weight loss, muscle wasting and weakness (Kim *et al* 2013) and accelerated linear growth in children (Schlesinger *et al* 1973). In hyperthyroidism there are additional non organ-specific effects related to this hypermetabolic and catecholamine-sensitised state (Ginsberg 1981).

The association between abnormal thyroid function and changes in bone are well established. Hypothyroidism in children curtails bone growth and maturation leading to short stature, growth arrest (in severe cases) and susceptibility to slipped epiphysis (Foley *et al* 2004). Bone healing is severely compromised in hypothyroidism (Murrray 1900). More recently there has been recognition of increased susceptibility to bone fracture in adults (even in subjects with corrected hypothyroidism (Vestergaard & Mosekilde 2002). Examination of hypothyroid epiphyses reveals disorganised chondrocyte columns and suboptimal quality of the cartilaginous matrix (Stevens *et al* 2000). In contrast, these parameters appear essentially normal (though temporally advanced) in hyperthyroidism (Stevens *et al* 2000, Freitas *et al* 2005). Hyperthyroidism accelerates bone growth and maturation (Segni & Gorman 20001, Buckler *et al* 1986) which in cases of severe congenital hyperthyroidism can cause craniosynostosis (Johnsonbaugh *et al* 1978). In children, hyperthyroidism can cause earlier epiphyseal closure, which *may* compromise final height (Rivkees *et al* 1988, Segni *et al* 2001). In adults, hyperthyroidism uncouples the bone remodelling process resulting in net bone loss (Fraser *et al* 1971). Bone loss reduces bone volume, strength, and increases susceptibility to fracture (Lee & Ananthakrishnan 2011). However, it appears that this increased fracture risk returns to near normal, with time, following correction of hyperthyroidism (Vestergaard & Mosekilde 2002).

Free thyroid hormones (FTHs) influence bone cell biology to a large extent. T3 has the following effects: it accelerates chondrocyte differentiation (Robson *et al* 2000), stimulates osteoblast activity and osteoblastogenesis (Bassett & Williams 2009), and stimulates osteoclast activity and osteoclastogenesis (although this effect is probably mediated indirectly via the osteoblast (Britto *et al* 1994)). Thyroid receptors (TR) are expressed on osteoblasts (TRα and TRβ (Xing *et al* 2012, O'Shea *et al* 2003) and differentiating chondrocytes (Ballock *et al* 1999, Williams *et al* 2009). It is less clear whether they are expressed on osteoclasts although TR mRNA has been extracted from osteoclastoma cells (Allain *et al* 1996) and multinucleated osteoclasts (Abu *et al* 2000). T4 and T3 have similar effects on cell biology although T3 is more potent (Gross & Pitt-Rivers 1953) and has ~15 times the affinity of T4 for TRβ1 (Lin *et al* 1990).

Historically the metabolic impact of thyroid diseases have been attributed to the effects of the FTHs. However, data to suggest an independent effect from the reciprocally and more significantly altered TSH value (occurring in common primary thyroid disorders) is mounting (Abe *et al* 2003, Fatourechi *et al* 2009, de Lloyd *et al* 2010).

The TSH receptor (TSHR) has been detected in a number of extra-thyroidal sites (including adipose tissue, bone and fibroblasts; reviewed in Davies *et al* 2002), suggesting it may have a functional or developmental role in these sites. Mesenchymal stem cells (MSC) express TSHR (Bagriacik *et al* 2012) with the potential to differentiate into different terminal cells and tissues that influence body composition, including fat, muscle, cartilage and bone. It has been hypothesised that TSHR activation may influence lineage-specific differentiation (de Lloyd *et al* 2010). This theory is supported by the observation that MSC TSHR activation encourages chondrocyte and possibly osteoblast differentiation (Baliram *et al* 2011, Bagriacik *et al* 2012). Interestingly, an inverse relationship between bone and fat differentiation has been observed (Ng & Duque 2010) that could be influenced by this pathway.

The presence of TSHR in bone has raised academic interest, particularly given the socioeconomic impact of osteoporotic fractures (estimated at ~2 billion pounds per year in the UK in 2007; British Orthopaedic Association, 2007). Many studies intent upon delineating the role of TSHR in bone have been performed. Abe *et al* (2003) genetically engineered mice to be heterozygous or homozygous TSHR knockouts. It was observed that TSHR knockout

animals had low bone mass (and areas of focal osteosclerosis) despite normal (heterozygous) or normalised (homozygous animals) FTH values. Cell culture analysis of the TSHR-deficient and haploinsufficient bone cells revealed accelerated osteoclastogenesis and a greater number of colony forming unit-osteoblasts than wild type (WT). This group hypothesised that these changes were a likely direct consequence of diminished TSHR expression in bone. Sampath *et al* (2007) subsequently reported that exogenously administered TSH (administered to ovariectomised, euthyroid rodents) improved bone architecture and strength. Experiments evaluating osteoclastogenesis *in vitro* suggested an inhibitory effect from TSH or TSH-stimulating antibodies (occurring in Graves' disease) (Ma *et al* 2011) supporting the notion of a direct TSHR-mediated effect. More recent TSHR knockout experiments revealed exaggerated bone loss in TSHR deficient mice in the context of hyperthyroidism, relative to WT (both groups were rendered hypothyroid prior to precipitating hyperthyroidism) (Baliram *et al* 2012). It was suggested that TSHR signalling in the context of an unmeasurable TSH may relate to a bone marrow derived functional TSHβ splice variant (Vincent *et al* 2009) upregulated by T4 (Baliram *et al* 2012). However the Bassett & Williams group have challenged the conclusions relating to genetically modified animal models and suggest that their physiological observations can be largely explained by altered FTH values and sensitivities (Bassett *et al* 2008).

In subclinical thyroid diseases (ScTD) FTH values are in the normal range whilst TSH is outside it. These cohorts may therefore lend themselves to the identification of TSH-specific effects (independent of FTHs). Subclinical hyperthyroidism (Shyper) has been associated with accelerated bone loss, particularly in post-menopausal women (Faber *et al* 1998, Mudde *et al* 1994, Kim *et al* 2015). Indeed, the Joint American Endocrine Societies support this observation by advocating consideration to treating Shyper in all postmenopausal women and subjects ≥65 years of age not on bone protective medication (Bahn *et al* 2011). By contrast, the effect of SH on bone is less clear. Consequently, at present the Joint Endocrine Societies make no recommendations regarding bone health management in SH.

Several observational studies exploring the TSH-bone mineral density (BMD) relationship describe this as positive (athough this is predominantly described across *euthyroid* cohorts; see Table 1.5). This was the observation of a Chinese/ Malaysian cross-sectional study on

men (Chin *et al* 2013) that described a positive correlation between heel quantitative ultrasound readings and TSH (R +0.08, p-value 0.04, n=753). The Rotterdam study evaluated older adults (>55 years of age) with *no history of thyroid disease* and described this relationship at the femoral neck (Van der Deure *et al* 2008). Kim *et al* (2006) evaluated postmenopausal *euthyroid* Korean women and reported lower BMD and higher rates of osteoporosis amongst women with low-normal TSH (versus high normal TSH). Mazziotti *et al* (2005) evaluated thyroidectomised women with iatrogenic Shyper (n=66) and revealed suppression of bone resorptive markers (C-telopeptide of type 1 collagen) and a rise in bone-specific ALP within 2 days of administering recombinant TSH, an effect specific to the postmenopausal women (n=28). Thus supported the notion that TSH may have antiresorptive effects on bone.

However, other studies contradict these views and, describe a negative relationship between TSH and BMD in SH (Polovina *et al* 2013, Lee *et al* 2010, Liang *et al* 2014, Nagata *et al* 2007), or no relationship at all (in Indian adults <50 years of age (Marwaha *et al* 2012). An observational study (n=1477) on perimenopausal Dutch women screened for osteoporosis (with no history of thyroid disease or osteoporosis) reported a negative association between BMD and T4 at the lumbar spine (R -0.02, 95% C.I (-0.04 to -0.004) but no BMD-TSH relationship (Van Rijn *et al* 2014). Thus, much uncertainty remains.

Polovina *et al* (2013) evaluated FRAX score (an estimate of 10 year risk of fracture) in postmenopausal SH women (n=82) (with no prior history of thyroid disease) versus matched euthyroid controls (n=51). They identified a higher FRAX score in SH women (6.50±4.58 versus 4.35±1.56; p-value 0.001), particularly those with autoimmune thyroid disease (ATD); although bone turnover markers (BTMs) were no different between the TPO +ve and -ve groups. Lee *et al* (2010) explored incident fracture prevalence in older subjects (≥65 years) who were euthyroid or had ScTD. Their data revealed an increased incidence of hip fracture in men, but not women with ScTD (multivariable-adjusted hazard ratio (MVHR) for men with SH (n=184/3567) was 2.31 (95% CI; 1.25-4.27), MVHR for men with Shyper (n=29/3567 adults) was 3.27 (95% CI, 0.99-11.30)). However, a recent meta-analysis (n>70,000) did not reveal an association between SH and incident fracture but did report this association for Shyper (Blum *et al* 2015).

Human studies have explored the BMD-TSHR subject area further by evaluating the influence of TSHR polymorphisms on BMD. The D727E polymorphism was presumably selected for study because of its high prevalence and its association with lower TSH values (Hansen *et al* 2007). This was thought to relate to increased TSHR sensitivity (Gabriel *et al* 1999) although this was not demonstrated in other studies (Nogueira *et al* 1999). The Peeters group (Van der Deure *et al* 2008) associated this polymorphism with increased femoral neck BMD (~+2%), whilst a Chinese study drew the opposite conclusion; reporting a higher prevalence amongst osteoporotic men (n=150) versus controls (n=150), (Liu *et al* 2012).

In summary, thyroid diseases impact on bone metabolism and body composition markedly. However, their influence in ScTD, particularly SH, remains unclear. The ScTD are not uniform pathologies but arise from an array of causes. These include inflammatory, destructive, substrate-mediated (i.e iodine deficiency or excess), autoimmune and genetic causes (such as TSH receptor mutations (TSHR-Ms). It might therefore be anticipated that the natural history and metabolic consequence of SH will depend on its cause. Given the high prevalence of SH (affecting ~4-9% of the adult population (Canaris *et al* 2000, Hollowell *et al* 2002)) and the adverse socioeconomic impact of osteoporosis I thought it would be helpful to explore the TFT-BMD relationship further in an SH cohort and to relate this to aetiology.

I have recruited and evaluated an adult, treatment naïve, SH cohort and screened all subjects for TSHR-Ms. Metabolic bone data, including a DXA scan (evaluating bone mineral density at the lumbar spine (LS) and hip), serum bone profiles (for calcium, phosphate and alkaline phosphatase (ALP)) and serum BTMs (CTX as the bone resorption marker and P1NP as the bone formation marker), and anthropometric data and medical histories were collected.

One of my primary objectives was to explore whether TSHR-M subjects (anticipated to be inactivating mutations in this cohort and genetically similar to Abe *et al*'s knockdown mice) were more prone to altered BMD or bone turnover (BT). However as I anticipated having too few mutation subjects to draw meaningful conclusions; in addition I would compare bone-related parameters in those with and without thyroid autoimmunity. This was to see whether aetiology of SH; *autoimmune* versus *non-autoimmune* causes might influence these parameters. The TSHR-M subjects and those with other genetic causes for SH were expected to reside predominantly in the TPO negative cohort (Alberti *et al* 2002).

I have also explored the relationship between BMD and TFT components (free-T4 (T4), free-T3 (T3) and TSH) across the cohort, with my particular interest being TSH. In addition these relationships were assessed according to subject' TSHR-mutation (primary objective) or polymorphism status (secondary objective) and according to their *FOXE1* polyalanine tract length (PTL) polymorphism status (secondary objective). Given that the *FOXE1* gene is involved in thyroid gland morphogenesis and polymorphisms in proximity to *FOXE1* have been associated with altered TFT values (Gudmundsson *et al* 2009, Medici *et al* 2011, Taylor *et al* 2015) an effect on bone parameters is feasible.

I will now proceed to provide and discuss the metabolic bone observations made in this heterogeneous, adult SH cohort.

4.2 DXA BMD scores in the SH cohort

BMD was evaluated across the cohort using Z-scores rather than T-scores; both are provided on standard DXA (Dual-energy X-ray absorptiometry) reports. A DXA report provides a score for each bone parameter assessed, in this case at the 1st to 4th lumbar vertebrae (L1, L2, L3 & L4), and an average value for the total lumbar spine (TLS). At the hip, scores were provided for the femoral neck (FN), the trochanter, the inter-trochanteric region and an average total hip value (TH).

DXA scores indicate the number of standard deviations (SDs) the patient's value lies above (+) or below (-) the mean value for a sex-matched, healthy, normal reference population. The *T-score* compares the patient's BMD against a reference population at the stage of *peak bone mass* (~30 years of age), whereas the Z-score compares the patient's value against an *age*-matched, healthy population (World Health Organisation (WHO) 2007b). Therefore, the Z-score is a useful way of comparing DXA values across a cohort of various ages (in this case; 20 - 71 years).

The T-score is the parameter used by the WHO in their definition of *low bone mass*. This was initially applied to women at or above the age of 50 years, although subsequently applied to men (WHO 2007b). According to this definition a T-score above one SD *below* the mean is *normal*, whereas a value between 1 to 2.49 SDs *below* the mean defines *osteopenia*. A value

2.5 SDs or more *below* the mean defines *osteoporosis* (WHO; 1994 and 2007b). It is estimated that the risk of hip fracture increases ~2.6 fold for every SD drop in hip BMD, and ~2.3 fold for every SD drop in vertebral BMD (Marshall *et al* 1996). Osteoporosis is also diagnosed on purely clinical grounds when a low energy fracture is sustained. Once osteoporosis is diagnosed it is generally advisable to treat it (when benefit outweighs risk) to reduce the risk of future fractures.

Figure 4.1 illustrates the proportion of the cohort with BMD T-scores in each of the 3 WHO SD categories; *normal*, *osteopenia,* and *osteoporosis*. This reveals that half the cohort have low BMD T-scores (~40% with osteopenia (n = 76) and ~12% with osteoporosis (n = 25)). However, when these data are evaluated using Z-scores (Figure 4.2); a quarter have low BMD for age (which I define as a Z-score less than 1 SD below the reference mean) and 2.5% (n = 5) have a BMD value greater than 2.5 SDs *below* the reference mean. As explained below, this represents a higher than expected rate of low BMD.

Figure 4.1: Pie chart illustrating the proportion of subjects in my cohort with DXA T-scores falling in to each of the three WHO categories

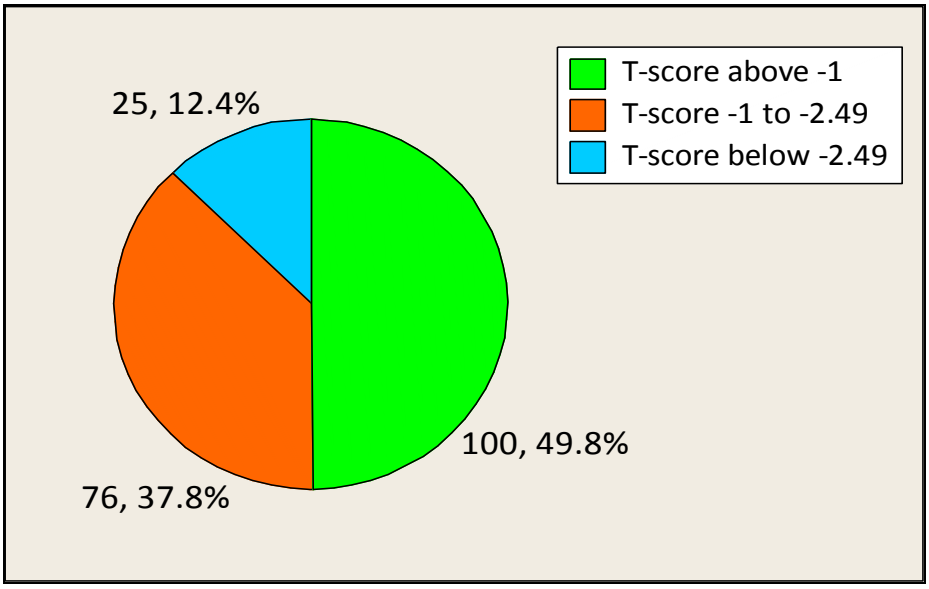


Figure 4.1 details the number and percentage of subjects falling into each of the three WHO categories. T-scores; above -1 (normal), between -1 to -2.49 (osteopenia), and below -2.49 (osteoporosis). The lowest T-score at any site determined the T-score category.

Figure 4.2: Pie chart illustrating the proportion of subjects in my cohort with DXA Z-scores in each of three BMD categories

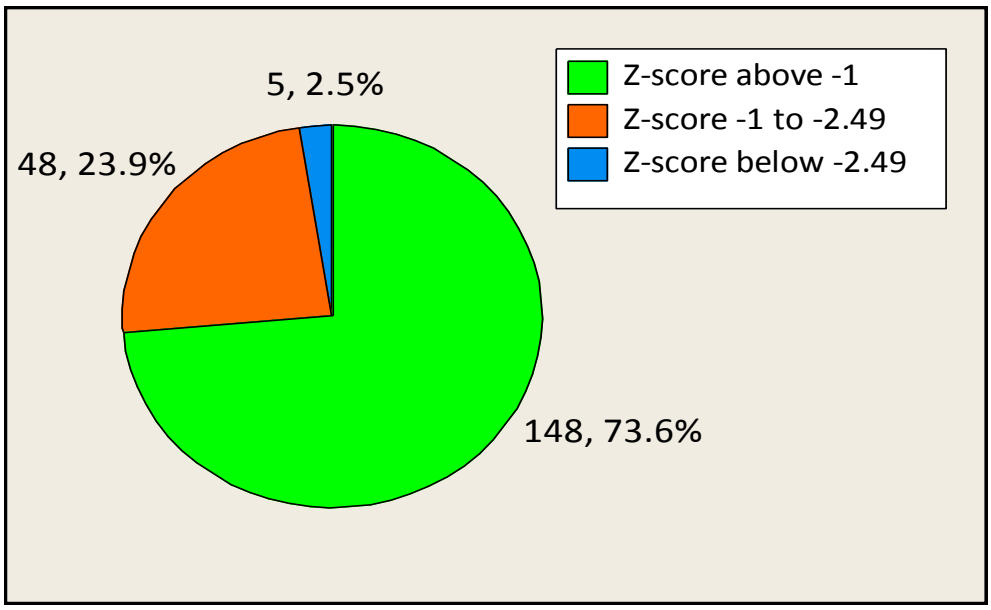


Figure 4.2 details the number and percentage of subjects falling into each of three DXA Z-score categories: above -1, between -1 and -2.49, and below -2.49. The lowest Z-score at any anatomical site determined the Z-score category.

The distribution of DXA BMD scores across this cohort at TLS and TH are represented by normal distributions (Figure 4.4). When I consider that a *perfect* normal population distribution would identify ~16% of the population with Z-scores >1 SD *below* the mean, and ~0.6% >2.5 SDs *below* the mean, it appears that low BMD is over-represented in my cohort; see figure 4.3. This is particularly so given that subjects with diagnosed osteoporosis or evident risk factors for osteoporosis were excluded from study participation (exclusions; section 2.1.2).

Further evaluation of the cohort's DXA scores (analysis not shown) revealed that low BMD (T-score *below* -1 (n=101 with complete data)) was over represented at the LS. Indeed 50% of this group (n = 50) had low BMD *specifically* affecting the LS (≥1 SD difference between LS and hip T-scores) versus 12% with low BMD specifically affecting the hip; an observation replicated when using the Z-score data. Further exploration revealed *minor discordance* (1 SD difference in T- or Z-scores between sites) in 89% of these subjects (55/62) and all those with *major discordance* (≥2 SD difference in T- or Z-scores between LS and hip) had lower BMD at the LS.

Figure 4.3: A perfect normal distribution detailing the percentage of a population expected within standard deviations of the mean

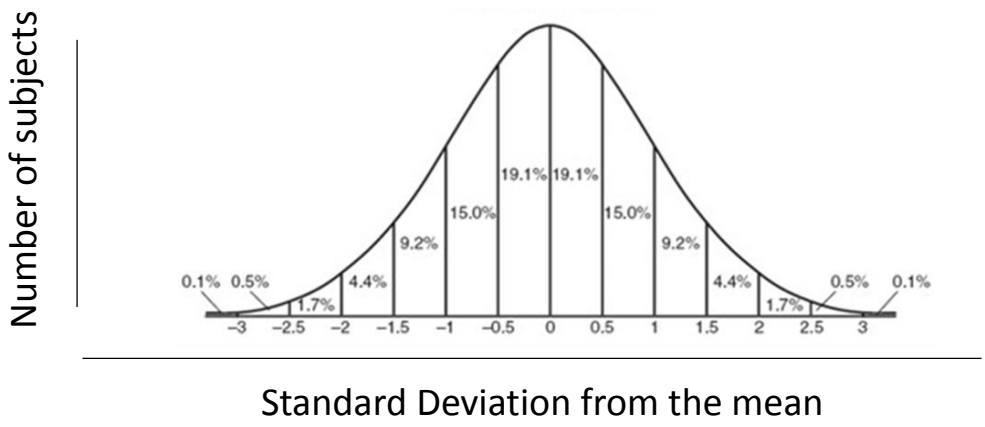
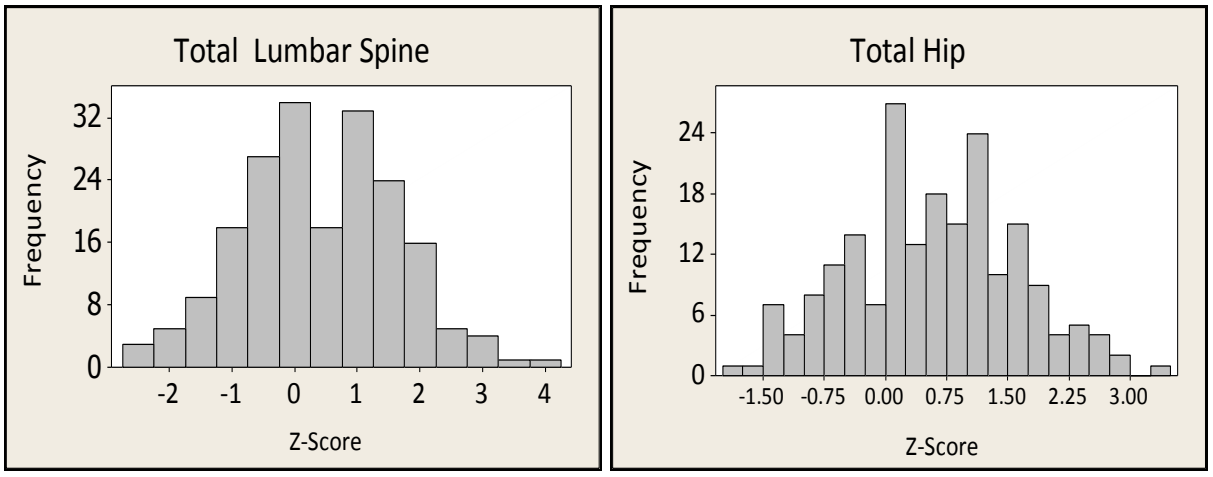


Figure 4.3 illustrates the percentage (%) of a perfect normal population falling into each SD sub-category. Copyright courtesy of Mathplanet: (http://www.mathplanet.com/education/ algebra - 2/quadratic-functions-and-inequalities /standard-deviation-and-normal-distribution)

Figure 4.4: Histograms illustrating the DXA Z-score distributions across the cohort at (a) Total Lumbar Spine and (b) Total Hip

(a) **(b)**



Total Lumbar Spine α= 0.42, SD =1.26 Total Hip α= 0.56, SD =1.0

Figure 4.4 displays the complete data of unadjusted DXA Z-score distributions at Total Lumbar Spine (a) and Total Hip (b) across the cohort. The mean Z-score (α) and SD are provided beneath each graph.

4.3 DXA Z-score against TSH at Lumbar Spine and Hip

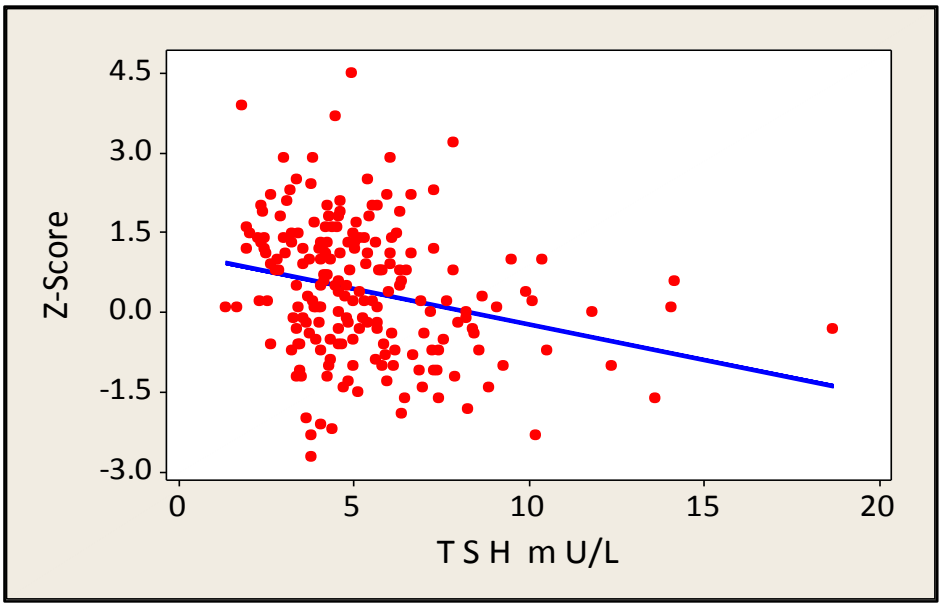
The TFT values provided in the figures and analyses that follow relate to those taken at study attendance. The TFT associations explored across the cohort so far revealed a negative association between TSH and T4 (Figure 3.17), and a small positive association between TSH and T3 (Figure 3.18). These associations should be borne in mind when considering the TSH-BMD relationships [Please see Section 2.19 for an explanation of the statistical parameters that are provided in the analyses that follow; p-values, confidence intervals (C.I), multiple regression analyses (MRAs), R^2 values, regression coefficients (R) and standard errors (SE)].

Figures 4.5 and 4.6 illustrate the unadjusted associations between TSH and BMD Z-scores (displaying the total cohort data, without removal of outliers) at the TLS (figure 4.5) and Femoral Neck (figure 4.6). Their respective regression equations are shown below. A negative relationship between Z-score and TSH at both anatomical sites was revealed.

Total Lumbar Spine Z-score	=	1.13 - 0.13 TSH,	p-value <0.001,	R^2 6.5%
Femoral Neck Z-Score	=	0.92 - 0.1 TSH,	p-value = 0.001,	R^2 5%

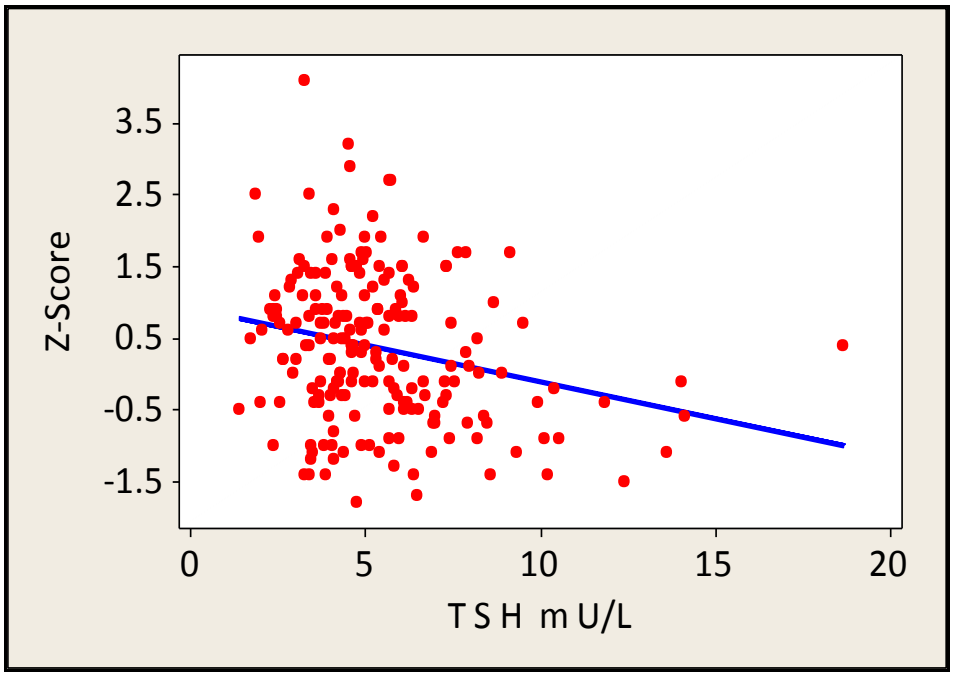
Table 4.1 details the regression coefficients (R) between TSH and Z-score at all anatomical sites evaluated by DXA. In general the *negative* relationship between TSH and Z-score is more pronounced at the LS than the hip.

Figure 4.5: Scattergraph illustrating the relationship between TSH and Total Lumbar Spine Z-score across the cohort



TSH values are shown on the x-axis and total lumbar spine Z-scores on the y-axis. Red dots represent participant's values and the blue line represents the best linear association between these parameters.

Figure 4.6: Scattergraph illustrating the relationship between TSH and Femoral Neck Z-score across the cohort



TSH values are shown on the x-axis and femoral neck Z-scores on the y-axis. Red dots represent study participant readings and the blue line represents the best linear relationship between these parameters.

Table 4.1: Regression coefficients between TSH and Z-score at each anatomical site evaluated by DXA

Region Evaluated	R	95% C.I.	R^2 (%)	p-value
Total Lumbar Spine	-0.13	-0.2, -0.06	7	<0.001
1st Lumbar Vertebrae	-0.125	-0.18, -0.05	6	<0.001
2nd Lumbar Vertebrae	-0.14	-0.21, -0.07	7.3	<0.001
3rd Lumbar Vertebrae	-0.13	-0.21, -0.05	5.3	0.001
4th Lumbar Vertebrae	-0.12	-0.18, -0.03	4.5	0.003
Total Hip	-0.07	-0.13, -0.02	3	0.014
Femoral Neck	-0.13	-0.13, -0.04	5.8	0.001
Inter-Trochanter	-0.06	-0.12, -0.003	2	0.04
Trochanter	-0.07	-0.13, -0.01	2.5	0.015

The anatomical regions evaluated by DXA are listed together with their corresponding Z-score-TSH regression coefficients (R), 95% Confidence Intervals (C.I), R^2 and p-values.

4.4 Multiple Regression Analyses

Single factor associations (depicted in Figures 4.5 and 4.6) are not the recommended way to evaluate complex relationships as other factors (known and unknown) may be influencing or explaining this relationship and are not being accounted for. Instead, relationships are more robustly evaluated by analyses that consider multiple parameters simultaneously, as is the case with *multiple regression analysis* (MRA).

In MRAs the contribution of each parameter within an equation is assessed, and its contribution towards that relationship *predicted* statistically. Although an MRA might suggest an association, this does not confirm a cause-effect relationship. This is because causation needs to be determined through prospective case-controlled studies where one variable at a time is altered and its impact determined. However, an MRA is a helpful tool to identify relationships that can be hypothesised to be inter-dependent. In many cases I have proceeded to evaluate relationships through a step wise parsimonious MRA. This enables the relationship between parameters of interest to be evaluated step by step as potential confounders are added in to the model. A consistent relationship between these parameters, despite the step-wise addition of confounders, allows greater confidence that this relationship is true.

One of the conditions of MRAs is that continuous data must be *normally* distributed (with appropriate transformation if necessary) and outliers removed, as the data and its interpretations thereafter may otherwise be misleading. These conditions of MRA are applied in every case where it is used.

4.4.1 MRAs of DXA Z-scores

These MRAs assess dichotomous as well as continuous variables. in this context the *affirmative status* of the binary parameter *in* the MRA is being assessed. The symbols used for the binary parameters are as follows; Smoking(1) for a >10 pack year smoking history; Alcohol(1) for *not* tee-total; Activity(1) for a *non-sedentary* lifestyle, TSHR-M for TSHR-mutation status, D727E or P52T for the respective TSHR polymorphism status, FOXE1(14) for homozygous FOXE1 PTL 14 status, TPO (+ve) for TPO seropositivity, and male(1) for male sex.

The association coefficient (R) for the *opposing* binary parameter (i.e. female instead of male) is the inverse of the association coefficient given.

Alcohol intake was assessed several different ways in these evaluations (i.e unit intake per week; heavy intake (>14 units Female, >21 units Male) versus non-heavy intake; consumer versus non-consumer, and in all evaluations an associations with Z-score was not revealed.

In the Z-score MRAs that follow, smoking history, alcohol history and T4 are not included. This is because no Z-score associations were shown, and the more variables included in the MRA the higher the chance of revealing false associations (Type 1 errors).

4.4.2 Total Lumbar Spine MRA

The parameters assessed in the TLS Z-score MRA include waist to hip ratio (WHR), activity status, male sex, TSH and T3 values. The MRA with its respective regression table are shown below.

TLS Z-score = -0.26 + 4.1 **WHR** + 0.32 **Activity(1)** - 0.8 **Male(1)** - 1.7$\log^{10}$ **TSH** - 0.4 **T3**

N = 196, R^2 = 18%

Table 4.2: Summarises the MRA evaluating Total *Lumbar Spine* Z-score against the predictors listed.

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-0.26	1.18	-2.6, +2.0	0.82
WHR	+4.1	1.14	+1.8, +6.3	<0.001
Activity (1)	+0.32	0.17	-0.01, +0.65	0.07
Male Sex (1)	-0.8	0.23	-1.25, -0.35	0.001
Log^{10} TSH mU/L	-1.7	0.45	-2.56, -0.8	<0.001
T3 pmol/L	-0.40	0.17	-0.73, -0.07	0.02

Table 4.2 details the Regression Coefficient (R) for each predictor assessed in the MRA evaluating TLS Z-score. The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

This MRA revealed the following TFT associations against TLS Z-score;-

- A negative correlation against (Log^{10})**TSH** (R; -1.7, p-value <0.001)
- A negative correlation against **T3** (R; -0.4, p-value 0.02)

This MRA revealed the following non-TFT associations against TLS Z-score;-

- A negative correlation against **male sex** (R; -0.8 , p-value 0.001)
- A positive correlation against **WHR** (R; +4.1, p-value <0.001)

4.4.3 Total Hip MRA

The parameters assessed in this MRA (evaluating TH Z-score) include WHR, activity status, male sex, TSH and T3 values. The MRA with its respective regression table are shown below.

TH Z-score = - 1.6 + 4.3 **WHR** + 0.06 **Activity (1)** - 0.8 **Male(1)** - 0.67 Log^{10}**TSH** - 0.2 **T3**
N=198, R^2 = 16%

Table 4.3: Summarises the MRA evaluating *Total Hip* Z-score against the predictors listed

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-1.6	1.0	-3.6, +0.36	0.1
WHR	+4.3	0.97	+2.4, +6.2	<0.001
Activity (1)	+0.06	0.15	-0.23, +0.35	0.69
Male(1)	-0.80	0.2	-1.2, -0.4	<0.001
Log^{10} TSH mU/L	-0.67	0.40	-1.5, +0.1	0.09
T3 pmol/L	-0.20	0.14	-0.07, +0.47	0.16

Table 4.3 provides the regression coefficients (R) for each predictor assessed in the MRA evaluating TH Z-score. The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

The TH Z-score MRA did not reveal associations with TFT components but did reveal the following associations;-

- A negative correlation with **male sex** (R -0.8, p-value 0.001)
- A positive correlation with **WHR** (R +4.3, p-value <0.001)

The MRAs evaluating TLS and TH Z-scores were subsequently re-evaluated substituting body mass index (BMI) for WHR which revealed slightly different associations. At both sites R^2 improved (18% to 24% at TLS, 16% to 36% at TH) and Activity became positively associated with Z-score (LS; R +0.4, p-value 0.01, TH; R +0.3, p-value 0.037). At TH, a negative association against T3 was now revealed (coefficient -0.35, p-value 0.005) (see Appendix 6, Table 1)

I subsequently evaluated how the correlation coefficient (R) between DXA Z-score and TSH changed with the addition of confounders using a stepwise parsimonious regression model at TLS and TH (Model 4.1).

Stepwise regression Model 4.1 revealed a small attenuation in R at TLS as confounders were added in to the model (over steps 1-4). This supports the hypothesis that TSH correlates independently and inversely against Z-score at TLS. The equivalent analysis at TH showed a more substantial fall in R as confounders were added in to the model with a final p-value falling short of statistical significance (0.09). This does not support an independent correlation between TSH and Z-score at TH, although alternatively the study may be underpowered to reveal this effect.

A further parsimonious stepwise regression analysis was performed *comparing* correlation coefficients for DXA Z-score and TSH, with DXA Z-score and T3. This was evaluated at TLS (Model 4.2) and then TH (Model 4.3). A Bonferroni corrected p-value of 0.025 was used here to define statistical significance given that the TSH and T3 correlations were compared. BMI was the build parameter used in these models.

Stepwise regression models 4.2 and 4.3 support the observation that TSH correlates negatively with Z-score at TLS (Model 4.2) (despite a diminution in the coefficient for TSH with addition of confounders (not seen for T3). Model 4.3 does not support an independent correlation between TSH and DXA Z-score at TH. Both models revealed a negative association between T3 and DXA Z-score at TH and TLS sites.

Model 4.1: Stepwise regression model exploring R between TSH and DXA Z-score at total lumbar spine & total hip; assessed singularly and subsequently adjusted for confounders

	Total Lumbar Spine				Total Hip		
N	**R**	**95% C.I.**	**p-value**	**N**	**R**	**95% C.I.**	**p-value**
	Step 1; crude (against TSH only)						
198	-1.9	(-1, -2.8)	<0.001	200	-0.87	(-0.1, -1.65)	0.03
	Step 2; adjusted for step 1 including T3						
198	-1.7	(-0.8, -2.6)	<0.001	200	-0.77	(0.02, -1.56)	0.057
	Step 3; adjusted for step 2 including male sex						
198	-1.65	(-0.75, -2.54)	<0.001	198	-0.72	(0.06, -1.5)	0.07
	Step 4; adjusted for step 3 including waist:hip ratio and non-sedentary lifestyle						
196	-1.68	(-0.8, -2.57)	<0.001	198	-0.67	(0.1, -1.42)	0.09

Model 4.2: Stepwise regression model evaluating R between total lumbar spine Z-score and TSH (firstly) then total lumbar spine and T3 (secondly); both are subsequently adjusted for confounders

	Adjusted for TSH				Adjusted for T3		
N	**R**	**95% C.I.**	**p-value**	**N**	**R**	**95% C.I.**	**p-value**
	Step 1; crude						
198	-1.9	(-2.8, -1)	<0.001	198	-0.54	(-0.88, -0.2)	0.002
	Step 2; adjusted for step 2 including activity status						
198	-1.93	(-2.8, -1.0)	<0.001	198	-0.54	(-0.9, -0.2)	0.002
	Step 4; adjusted for step 3 including log^{10} BMI						
198	-1.73	(-2.6, -0.9)	<0.001	196	-0.67	(-1.0, -0.36)	<0.001
	Step 5; adjusted for step 4 including male sex						
198	-1.61	(-2.5, -0.76)	<0.001	196	-0.6	(-0.9, -0.3)	<0.001
	Step 6; adjusted for step 4 including T3 or TSH respectively						
198	-1.36	(-2.2, -0.5)	0.002	196	-0.5	(-0.8, -0.2)	0.002

Models 4.1 and 4.2 detail the number of subjects in the analysis (N), the correlation coefficients (R) between either TLS Z-score and TSH, and TH Z-score and TSH (Model 4.1); or between TLS Z-score and log^{10}TSH, and TLS Z-score and T3 (Model 4.2). The 95% confidence interval (C.I.) and p-valus at each step are provided. The minus (-) or plus (+) signs indicates the direction of the association.

Model 4.3: **Stepwise regression model exploring R between total hip Z-score and TSH (firstly) and total hip Z-score and T3 (secondly); both are subsequently adjusted for confounders**

	Adjusted for TSH				Adjusted for T3		
N	**R**	**95% C.I.**	**p-value**	**N**	**R**	**95% C.I.**	**p-value**
	Step 1; crude						
200	-0.87	(-1.65, -0.1)	0.03	200	-0.26	(-0.55, +0.02)	0.07
	Step 2; adjusted for step 2 including activity status						
200	-0.8	(-1.6, -0.02)	0.045	200	-0.27	(-0.6, +0.02)	0.063
	Step 3; adjusted for step 3 including log^{10} BMI						
198	-0.54	(-1.22, +0.14)	0.12	200	-0.45	(-0.7, -0.2)	<0.001
	Step 4; adjusted for step 4 including male sex						
198	-0.42	(-1.1, +0.23)	0.20	200	-0.37	(-0.6, -0.13)	0.003
	Step 5; adjusted for step 4 including T3 or TSH respectively						
198	-0.25	(-0.9, +0.4)	0.46	198	-0.35	(-0.6, -0.1)	0.005

Model 4.3 details the number of subjects used in the analysis (N), the correlation coefficient (R) between TH Z-score and TSH, or TH Z-score and T3. The 95% confidence interval (C.I.) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

4.4.4 DXA Z-score associations according to aetiology of SH with respect to TPO antibody status and thyroid genetic data

In this section the aetiology of SH was assumed to be autoimmune thyroid disease (ATD) in subjects bearing positive (+ve) TPO antibodies, and assumed to be due to TSHR-Ms where these were identified.

Associations between Z-score and TSHR-polymorphism status (D727E and P52T) and FOXE1 PTL polymorphism status are assessed in this chapter (see introduction; section 4.1).

The following figures illustrate the relationship between Z-score and TPO+ antibody status (Figure 4.7a-c), TSHR gene sequence (TSHR mutation, TSHR polymorphisms (D727E or P52T) and standard TSHR sequence (Figure 4.8a-c) and FOXE1 PTL polymorphism status (Figure 4.9a-c). The statistical evaluations that relate to these figures are provided in section 4.4.6.

Figure 4.7 a-c: DXA Z-score according to TPO antibody status at (a) Total Lumbar Spine, (b) Total Hip and (c) Femoral Neck

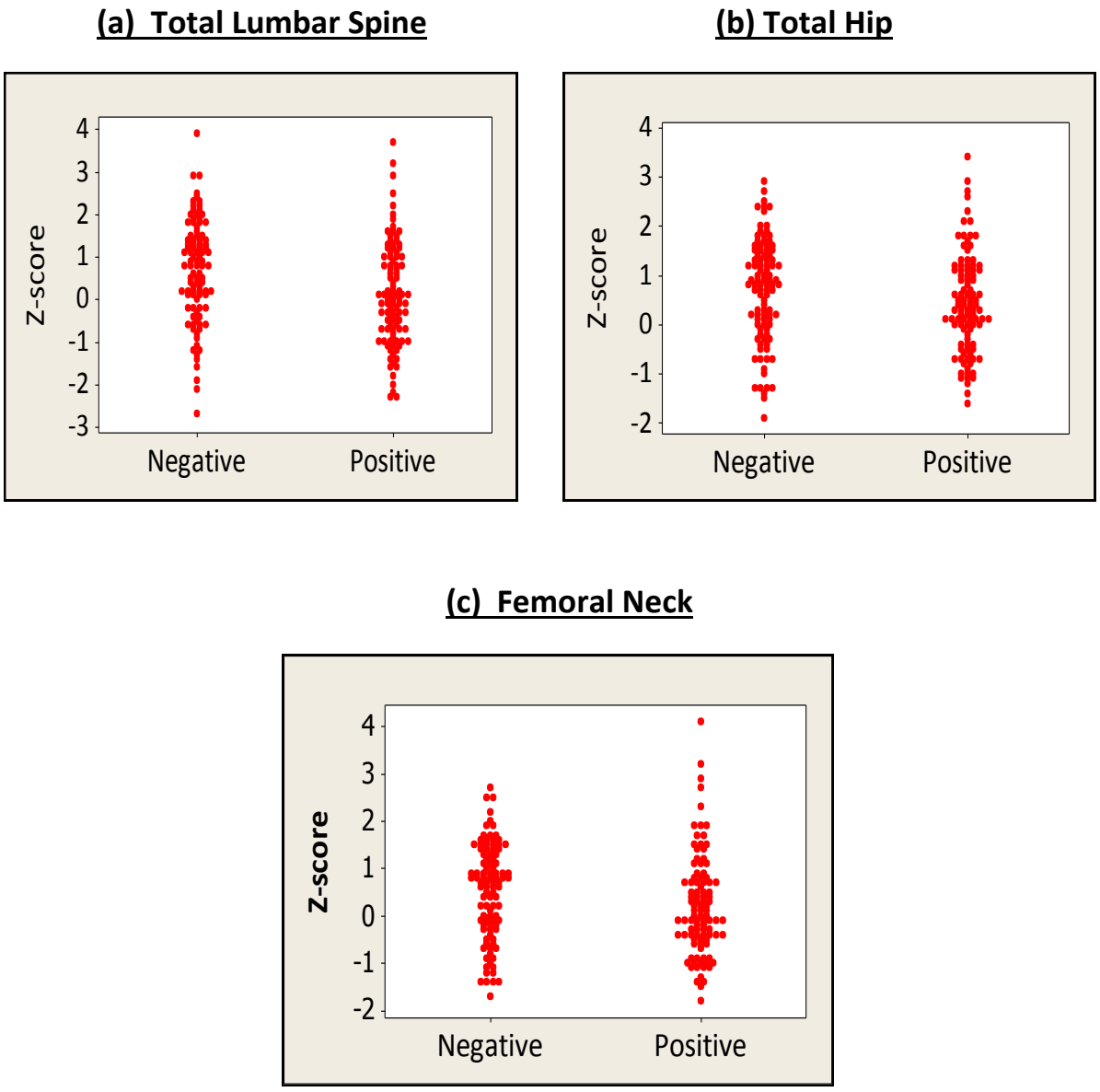


Figure 4.7 depicts DXA Z-scores according to TPO antibody status (positive or negative) at each of three anatomical sites: a) Total Lumbar Spine, b) Total Hip, c) Femoral Neck. The red dots represent individual's values that collectively indicate the distribution within the cohort.

Figure 4.8 a-c: **DXA Z-score according to TSHR sequence status at (a) Total Lumbar Spine, (b) Total Hip and (c) Femoral Neck**

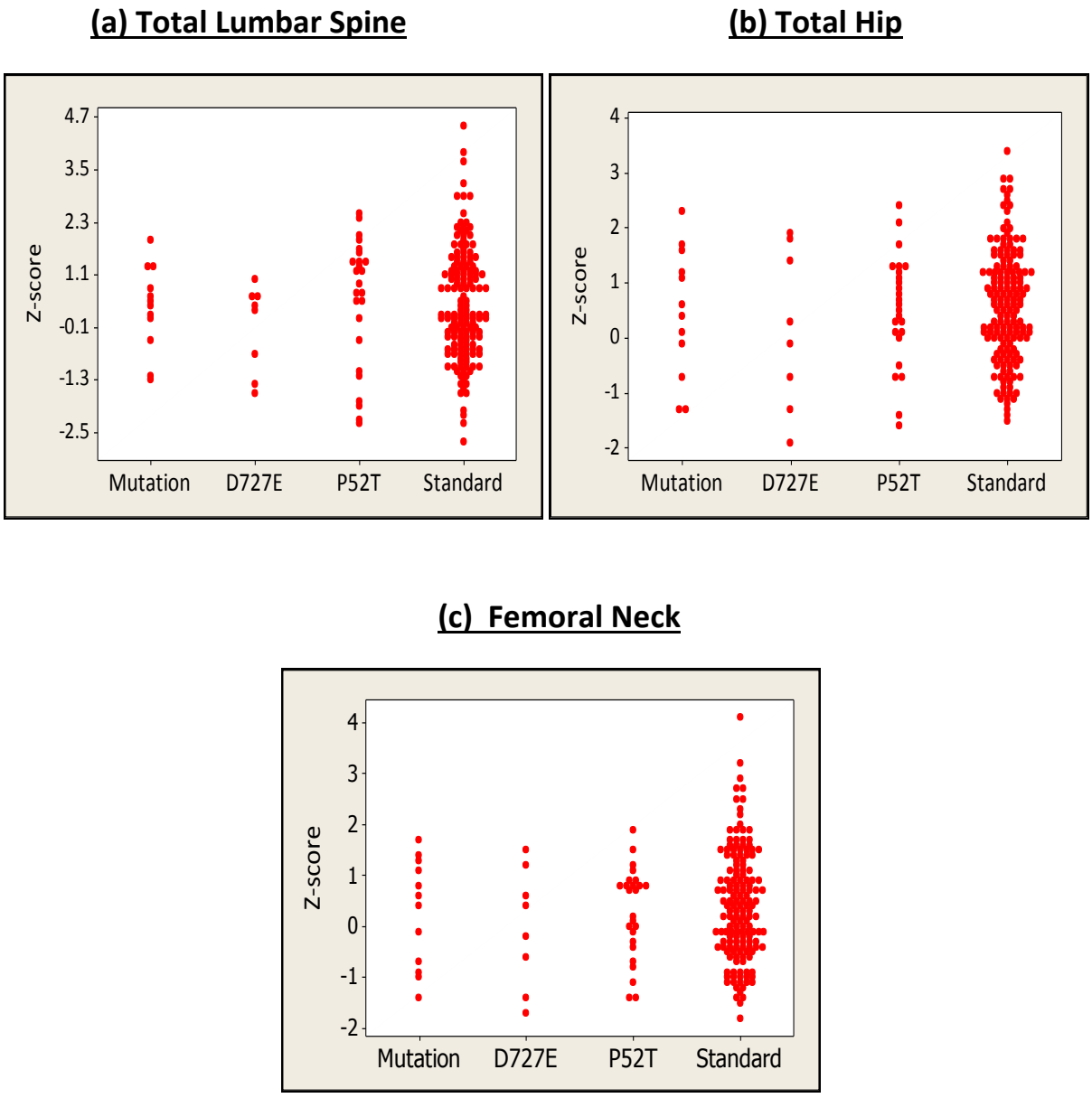


Figure 4.8 depicts DXA Z-scores according to TSHR gene sequence at each of three anatomical sites: a) Total Lumbar Spine, b) Total Hip, c) Femoral Neck. The TSHR sequence categories are those with mutations, polymorphisms (D727E or P52T) or standard sequence. The red dots represent individual's values that collectively indicate the distribution within the cohort.

Figure 4.9 a-c: **DXA Z-scores according to *FOXE1* PTL polymorphism status at a) Total Lumbar Spine, b) Total Hip, c) Femoral Neck**

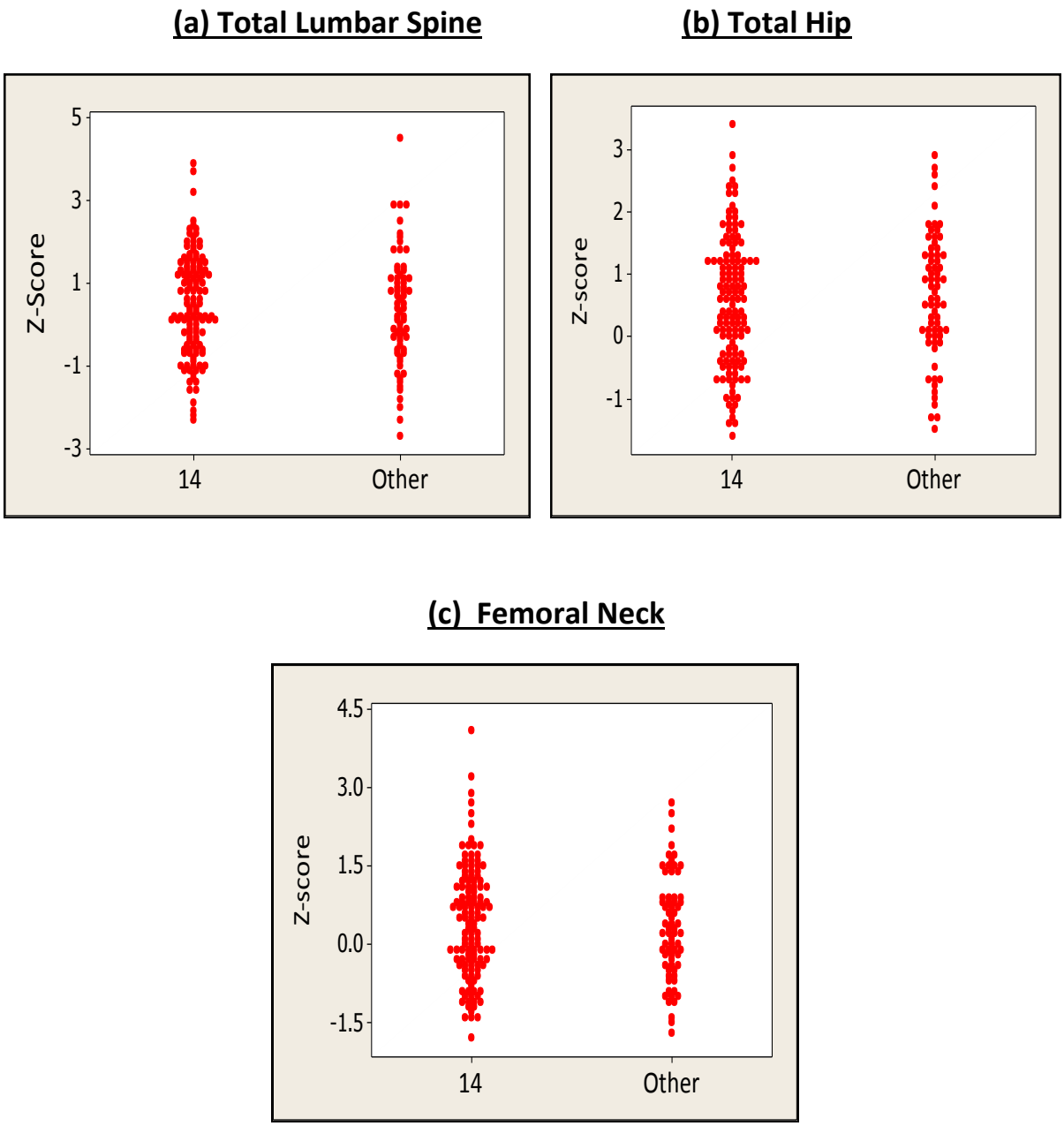


Figure 4.9 depicts DXA Z-scores according to FOXE1 PTL polymorphism status categorised as 14/14 (14) or 'other' polyalanine tract length combinations. These data are provided at a) Total Lumbar Spine, b) Total Hip and c) Femoral Neck. The red dots indicate individual's values that collectively indicate the distribution within the cohort.

4.4.5 Thyroid function, anthropometric and bone data according to TPO antibody status

Half my study cohort were TPO antibody positive (+ve) (104/208) and half TPO negative (-ve). Table 4.4 compares the biochemical and clinical characteristics of participants according to their TPO status.

Table 4.4: Biochemical and clinical characteristics of the cohort according to TPO antibody status

Variable	TPO +ve	TPO -ve	$\bar{x}$ difference	95% C.I	p-value
% male	16%	34%	-16%	-0.29, -0.06	0.003
$\bar{x}$ **Age (years)**	49.2	54.4	-5.2	-8.7, -1.7	0.004
$\bar{x}$ **BMI**	27.5	29.7	-2.2	-4, -0.37	0.02
$\bar{x}$ **WHR**	0.84	0.88	-0.04	-0.07, -0.02	0.001
$\bar{x}$ **proportion active**	0.6	0.43	+0.16	+0.03, +0.3	0.02
$\bar{x}$ **TSH (mU/L)**	5.8	4.8	+1	+0.34, +1.7	0.002
$\bar{x}$ **T4 (pmol/L)**	12.7	13.6	-0.9	-1.3, -0.5	0.001
$\bar{x}$ **T3 (pmol/L)**	4.05	4.13	-0.08	-0.2, +0.06	0.24
$\bar{x}$ **Ca^{2+} (mmol/L)**	2.34	2.35	-0.02	-0.03, +0.01	0.28
$\bar{x}$ **Phosphate (mmol/L)**	1.1	1.09	+0.01	-0.03, +0.04	0.7
$\bar{x}$ **ALP (IU/L)**	75	86	-11	-20, -0.7	0.035
$\bar{x}$ **CTX (µg/L)**	0.234	0.228	+0.007	-0.02, +0.04	0.7
$\bar{x}$ **P1NP (µg/L)**	41	43	-2	-7.1, +3.2	0.46

Table 4.4 lists parameters whose mean ($\bar{x}$) proportion, %, or value was compared between the TPO +ve and -ve groups. The $\bar{x}$ difference, 95% confidence intervals (C.I), and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

Table 4.4 reveals that the TPO +ve group differed from the -ve group in the following respects:

- A greater proportion of women (84% versus 66%).
- Younger mean age (49 years versus 54 years).
- Lower mean T4s (12.7 versus 13.6pmol/L) and higher mean TSHs (5.8 versus 4.8mU/L).
- Lower mean BMIs and WHRs.
- Fewer subjects with a sedentary lifestyle.
- Lower mean alkaline phosphatase (ALP) values (75 versus 86 IU/L).

The following factors were not appreciably different:

- Mean T3s.
- Mean corrected calcium and phosphate values.
- Mean BTMs; CTX (bone resorption marker), and P1NP (bone formation marker).

4.4.6 Multiple regression analyses evaluating DXA Z-scores against TPO antibody status and the thyroid genetic data

In the MRAs that follow, the parameters assessed against Z-score are as previously reported (in section 4.4.2) but now include TPO antibody status and the thyroid genetic data; TSHR-M, TSHR polymorphism status (D727E or P52T) and *FOXE1* PTL(14) status.

The MRAs evaluating DXA Z-score at TLS and TH are shown below with their respective regression tables (tables 4.5 and 4.6 respectively).

Z-Score at Total Lumbar Spine = 0.8 + 3.4 WHR + 0.39 Activity - 0.86 Male(1) - 1.4 Log^{10} TSH - 0.5 T3 - 0.65 **TPO(+ve)** - 0.27 **TSHR-M** - 0.3 **D727E** + 0.05 **P52T** - 0.23 **FOXE1 PTL(14)**

N = 191, R^2 = 25%

Z-Score at Total Hip = - 0.7 + 3.5 WHR + 0.08 Activity - 0.8Male - 0.7 Log^{10}TSH - 0.2 T3 - 0.3 **TPO (+ve)** - 0.07 **TSHR-M** - 0.2 **D727E** + 0.01 **P52T** -0.1 **FOXE1 PTL(14)**

N = 193, R^2 = 17%

Table 4.5: Summarises the MRA evaluating Total Lumbar Spine Z-score against the predictors listed (including TPO status and the genetic data).

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+0.8	1.2	-1.6, +3.2	0.5
WHR	+3.4	1.15	+1.15, +5.7	0.004
Activity (1)	+0.39	0.18	+0.04, +0.74	0.03
Male (1)	-0.86	0.23	-1.31, -0.41	<0.001
Log^{10} TSH mU/L	-1.3	0.46	-2.2, -0.4	0.004
T3 pmol/L	-0.5	0.17	-0.8, -0.17	0.003
TPO (+ve)	-0.65	0.18	-1.0, -0.3	**<0.001**
TSHR-M	-0.27	0.36	-1.0, +0.4	0.45
D727E	-0.32	0.44	-1.2, +0.5	0.5
P52T	+0.05	0.25	-0.44, +0.5	0.83
FOXE1 (14)	+0.23	0.17	-0.1, +0.6	0.19

Table 4.6: Summarises the MRA evaluating Total Hip Z-score against the predictors listed (including TPO status and the genetic data).

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-0.7	1.1	-2.9, 1.5	0.5
WHR	+3.5	1	+1.5, +5.5	0.001
Activity (1)	+0.08	0.15	-0.2, +0.4	0.6
Male (1)	-0.8	0.20	-1.2, -0.4	<0.001
Log^{10}TSH (mU/L)	-0.65	0.4	-1.4, +0.13	0.1
T3 (pmol/L)	-0.2	0.15	-0.5, +0.09	0.15
TPO (+ve)	-0.3	0.15	-0.6, 0.0	**0.05**
TSHR-M	-0.07	0.31	-0.7, +0.5	0.81
D727E	-0.22	0.4	-1, +0.6	0.56
P52T	-0.01	0.21	-0.42, +0.4	0.95
FOXE1 (14)	-0.1	0.14	-0.4, +0.2	0.5

Tables 4.5 and 4.6 provide the regression coefficients (R) for each predictor assessed in the MRA evaluating TLS Z-score or TH Z-score respectively. The corresponding standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

The MRAs displayed in Table 4.5 and 4.6 reveal the following associations:

- TPO positivity is negatively associated with Z-score at TLS (coefficient -3.6, p-value <0.001) and has a borderline negative association at TH (coefficient -0.3, p-value 0.05). There were no associations with the polymorphisms (TSHR-M, TSHR-D727E or P52T or FOXE1 PTL (14) status).

- The TPO-Z-score data is illustrated in Figure 4.7a-c. This suggests that a greater proportion of the TPO +ve cohort have Z-scores in the lower half of the Z-score distribution. By contrast, the equivalent graphs for the *TSHR* gene sequence data (Figure 4.8) and *FOXE1* PTL polymorphism data (Figure 4.9) do not depict obvious associations (although the numbers in these distributions are less).

The relationship between Z-score and TPO antibody status at TH and TLS was subsequently evaluated in parsimonious stepwise regression analyses (Model 4.4).

Model 4.4 reveals a consistent negative association between TPO positive status and Z-score at TLS. Whereas at TH; no association is revealed until the final step that is of borderline significance. Given the discrepancy between associations at the 5th and final step this is likely to represent a chance finding. The model therefore supports a negative relationship between TPO positivity and Z-score at TLS only.

The relationship between Z-score and TPO status was subsequently explored, for further verification using a bootstrap analysis (with thanks to Dr Pete Taylor Cardiff University). This statistical method differs from MRAs in that it evaluates an extensive number of random samples within the data set (1000) that are combined to provide the final association coefficients and significance scores. The bootstrap analyses are summarised in Table 4.7 (TLS) and Table 4.8 (TH). These analyses support the observation of earlier MRAs indicating a negative relationship between TPO positivity and Z-score at TLS but not TH.

Model 4.4: Stepwise regression model exploring R between DXA Z-score and TPO antibody positivity assessed at total lumbar spine and total hip singularly, and subsequently adjusted for confounders.

	Total Lumbar Spine				Total Hip		
N	**R**	**95% C.I.**	**p-value**	**N**	**R**	**95% C.I.**	**p-value**
	Step 1; crude (against TPO status only)						
198	-0.56	(-0.9, -0.22)	0.001	198	-0.25	(-0.54, +0.33)	0.09
	Step 2; adjusted for step 1 including waist:hip ratio						
198	-0.54	(-0.9, -0.19)	0.003	198	-0.17	(-0.5, +0.12)	0.26
	Step 3; adjusted for step 2 including male sex						
196	-0.6	(-0.95, -0.26)	0.001	198	-0.23	(-0.5, +0.05)	0.11
	Step 4; adjusted for step 3 including TSH and T3						
196	-0.52	(-0.85, -0.18)	0.003	198	-0.2	(-0.5, +0.09)	0.18
	Step 5; adjusting for step 4 including activity status						
196	-0.56	(-0.9, -0.2)	0.001	198	-0.21	(-0.4, +0.1)	0.16
	Step 6; adjusting for step 5 and the genetic data (D727E, TSHR-M, P52T & FOXE1(14))						
191	-0.65	(-1, -0.3)	<0.001	193	-0.3	(-0.6, +0.0005)	0.05

Model 4.4 details the number of subjects in the analysis (N), the correlation coefficient (R) between TLS or TH Z-scores, and TPO positive status. The 95% confidence intervals (C.I.) and p-values at each step are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

Table 4.7: Bootstrap analysis evaluating total lumbar spine Z-score.

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+0.34	1.11	-1.8, +2.5	0.76
TP0 (+ve)	-0.56	0.17	-0.88, -0.23	0.001
Male (1)	-0.88	0.24	-1.35, -0.4	<0.001
WHR	+3.60	1.0	+1.6, +5.6	<0.001
Log^{10} TSH mU/L	-1.38	0.42	-2.2, -0.56,	0.001
T3 pmol/L	-0.43	0.17	-0.77, -0.9	0.013
Activity (1)	+0.38	0.18	+0.03, +0.7	0.03

Table 4.8: Bootstrap analysis evaluating total hip Z-score.

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-1.41	1.01	-0.56, +3.3	0.16
TP0 (+ve)	-0.21	0.14	-0.5, +0.06	0.13
Male (1)	-0.83	0.2	-0.4, -1.2	<0.001
WHR	+4.09	1.0	+2.1, +6	<0.001
Log^{10} TSH (mU/L)	-0.56	0.4	-0.2, +0.3	0.16
T3 (pmol/L)	-0.21	0.14	-0.5, +0.06	0.5
Activity (1)	+0.09	0.14	-0.18, +0.36	0.19

Tables 4.7 and 4.8 provide bootstrap analysis for the TLS- and TH Z-score analyses respectively. For each predictor variable its regression coefficient (R), standard error (SE), 95% confidence interval (C.I) and p-values are given. The minus (-) or plus signs (+) indicate the direction of the association.

4.5 In-cohort matching to compare the TSHR mutation and control subjects

The number of mutation patients in this study (n = 12) was extremely low and insufficient to demonstrate an impact on bone parameters using MRA alone.

Given this limitation I felt it would be worthwhile looking at this subgroup using in-cohort matching analysis. For this analysis I selected three control subjects for every mutation subject. All controls had standard TSHR sequence and were matched in the first instance for sex and TPO antibody status. Thereafter controls were matched as closely as possible for age, BMI and TSH values (at study attendance).

The matched data for the mutation and control subjects are provided in Table 4.10, and compared in Table 4.9. This revealed no significant differences.

Table 4.9: Comparison of matched data between mutation and control subjects

Parameter	Mean value	Difference	95% C.I	p-value
Age Mutation	49.8 years	0.53 years	-7.99, +9.05	0.9
Age Control	49.3 years			
BMI Mutation	27.8 kg/m^2	-0.35 kg/m^2	-3.88, +3.18	0.84
BMI Control	28.1 kg/m^2			
TSH Mutation	5.06 mU/L	0.14 mU/L	-0.880, +1.15	0.78
TSH Control	4.92 mU/L			

The mean values for each group, their differences, associated confidence intervals (C.I) and p-values for the difference are provided. The minus (-) or plus signs (+) indicate the direction of the difference.

The bone data comparing the mutation and control groups are provided in Table 4.11. Although this revealed a marginal reduction in TLS BMD amongst the mutation subjects (of borderline significance; difference -0.07, p-value 0.05) this did not tally with T-score differences and is unlikely to represent a true difference.

Another difference of borderline significance (and previously recognised) is that the mutation carriers had lower T3s than controls (difference 0.03, p-value 0.046).

The percentage body fat (%BF) and WHRs were not different between the groups (although subjects *were* matched for BMI).

Table 4.10: Matched data on TSHR-mutation and control subjects.

Mutation subjects				Controls			
Study Number	Age (years)	BMI (kg/m^2)	TSH (mU/L)	Study Number	Age (years)	BMI (kg/m^2)	TSH (mU/L)
3	34	17.3	3.4	77	22	21.9	6.11
				83	20	21	3.37
				83	20	21	3.37
22	40	29.2	4.88	186	39	28.9	5.41
				205	47	25.8	4.12
				208	44	28.2	4.82
46	61	24.5	7.66	137	65	32.5	7.31
				160	63	27.2	7.3
				179	64	24.9	4.94
84	44	32.3	4.65	14	38	25.4	6.68
				145	47	39.8	5
				153	42	31.4	4.58
111	66	30.8	5.66	67	67	39.8	5.72
				80	65	36.6	5.96
				146	69	31.7	4.07
119	52	25.5	4.83	29	52	22.5	5.44
				31	53	24.3	3.86
				55	55	22.7	5.03
177	58	38.1	2.88	15	59	28.9	3.04
				39	53	34.8	3.21
				53	57	29	2.28
32	43	26.4	3.42	58	42	23.5	4.88
				156	37	37	4.02
				159	33	33	5
78	38	26	6.16	5	52	29.8	5.03
				9	25	20	6.7
				159	33	27.9	5
99	63	29.4	5.95	17	66	31.4	5.37
				18	68	29.2	5.53
				173	62	30.7	6.32
148	38	27	7.01	1	47	28.3	4.6
				58	42	23.5	4.88
				96	37	23.1	7.16
172	61	26.9	4.18	19	66	27	4.09
				141	58	25.6	4.1
				191	64	26.5	4.23

Table 4.10 displays subject study numbers, ages, BMIs and TSH values for mutation subjects and their 3 respective controls. Red font represents female subjects and blue represents male subjects. Subject '83' is used twice as a control for mutation subject 3 due to insufficient matches for this young woman (BMI 17; normal 18.5-24.9).

Table 4.11: Comparative data on TFTs, bone and build parameters between the mutation and control subjects.

Parameter	Mutation mean	Control mean	Mean difference	95% C.I	p-value
T4 (pmol/L)	13.59	13.43	+0.16	-0.76, +1.1	0.72
T3 (pmol/L)	3.85	4.17	-0.32	-0.64, -6 x10^{-3}	**0.046**
BMD TLS (g/cm^2)	1.02	1.09	-0.07	-0.14, +8x10^{-4}	**0.05**
BMD TH (g/cm^2)	0.96	1.0	-0.04	-0.13, +0.05	0.36
BMD femoral neck (g/cm^2)	0.81	0.87	-0.06	-0.13, +9x10^{-3}	0.08
T-score TLS	-0.32	0.19	-0.5	-1.2, +0.19	0.15
T-score TH	-0.01	0.36	-0.36	-1.04, +0.31	0.27
T-score femoral neck	-0.48	-0.09	-0.4	-1.05, +0.27	0.23
Log^{10} P1NP	1.59	1.59	+0.006	-0.14, +0.13	0.93
Log^{10} CTX	-0.76	-0.65	-0.1	-0.25, +0.05	0.18
% body fat	32.8	33.3	-0.5	-7.9, +6.9	0.89
WHR	0.88	0.86	+0.02	-0.05, +0.09	0.58

The differences between mean values for the parameters listed are provided with their 95% confidence intervals (C.I) and p-values. The minus (-) or plus signs (+) indicate the direction of the difference.

4.5.1 Evaluation of mutation subjects against the rest of the cohort *not* matching for build

This analysis was performed in case the matching process in section 4.5 concealed true build differences (BMI, %BF, WHR & BMD) between the groups. In this analysis the mutation group were matched against standard TSHR sequence controls (as closely as the cohort allowed) for the parameters listed below. This enabled 12 mutation subjects to be compared against 56 controls.

Note that mutation subject 3 was included in this analysis although her BMI was 17 making her an outlier in the cohort.

- **Age** TSHR-M: 34 - 68 years Controls: 36 - 68 years
- **TSH** TSHR-M: 2.88-7.66mU/L Controls: 2.8-7.3mU/L
- **Sex ratio** TSHR-M: 5/12 Male Controls: 21/56 Male
- **TPO status** All TPO antibody negative
- **Comorbidities** No insulin users in either group
- **Activity** No self-reported 'very active' subjects in either group

Table 4.12 displays the comparative data between the mutation and control groups for the matched parameters. Aside from a higher mean age amongst the control group (56 versus 50 years), which fell short of a statisticaly significant difference (p-value 0.09); TSH values, sex ratios and the proportion of 'non-sedentary' subjects were similar across the groups.

Table 4.12: Compares *matched* parameters between the mutation and control groups

Parameter	Mean value		Mean Difference	95% C.I	p-value
	Mutations	Controls			
Age (years)	50	56	-6	-14.08, +1.14	0.09
Proportion male	0.4	0.38	+0.02	-0.3, +0.39	0.8
TSH (mU/L)	5.04	4.68	+0.36	-0.6, +1.3	0.4
% Active (non-sedentary)	0.58	0.43	+0.16	-0.19, +0.50	0.36

Differences between mean values for the matched-parameters are provided with their 95% confidence intervals (C.I) and p-values. The minus (-) or plus signs (+) indicate the direction of the difference.

The mutation and control groups were compared for a range of parameters (as shown in table 4.13) and no build differences were apparent. The observation of a lower T3 amongst the mutation subjects was upheld.

The comparative build data are presented graphically in figures 4.10 (BMI) and 4.11 (DXA Z-scores). DXA Z-scores rather than T-scores were used on account of the small age discrepancy between the groups.

Figure 4.10: BMI of mutation and control subjects

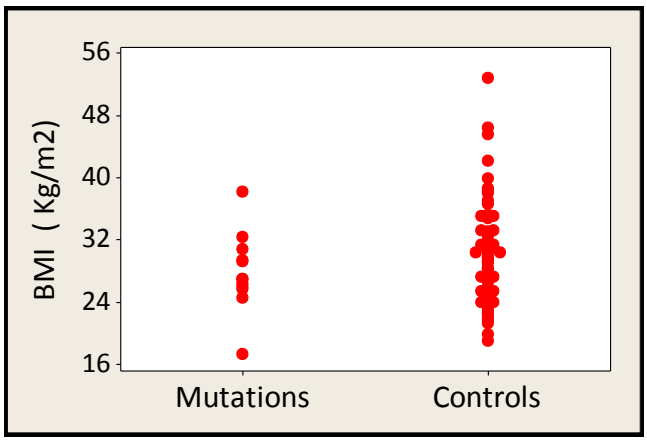
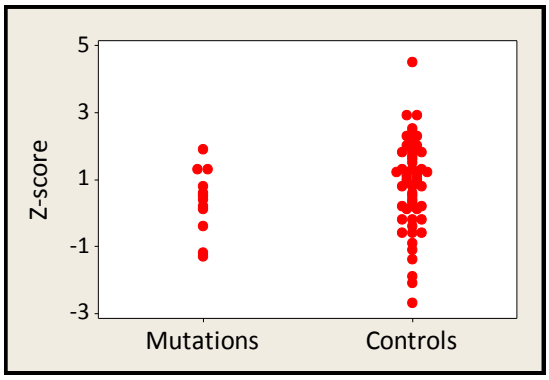
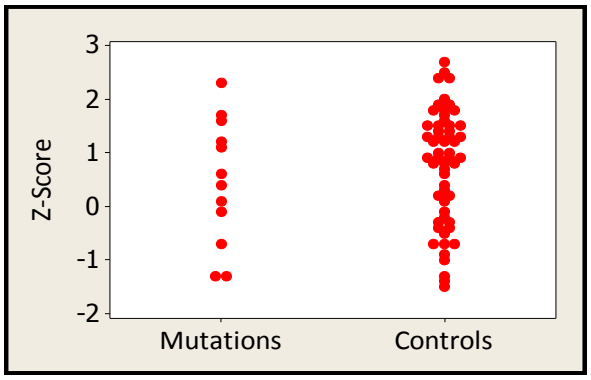


Figure 4.10; depicts the BMI value plots for the mutation and control subjects. The plots collectively indicate the BMI distribution across each group.

Figure 4.11: DXA Z-scores of Mutation and Control subjects at (a) Total Lumbar Spine, (b) Total Hip, and (c) Femoral Neck

(a) Total Lumbar Spine **(b) Total Hip**





(c) Femoral Neck

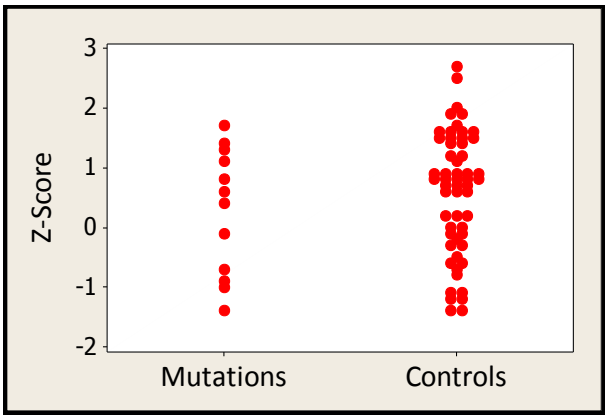


Figure 4.11. Depicts the DXA Z-score plots for the mutation and control subjects. The plots collectively illustrate the Z-score distribution within each group.

Table 4.13: Comparing mutation and control groups for parameters that include build.

Parameter	Mean values Mutations, Controls		Difference	95% C.I	p-value
BMI (kg/m^2)	27.8	30.2	-2.4	-6, +1.15	0.17
% Body Fat	32.8	36.3	-3.5	-10.8, +3.8	0.32
WHR	0.89	0.88	+0.005	-0.07, +0.06	0.9
T3 (pmol/L)	3.8	4.2	-0.4	-0.67, -0.05	**0.024**
T4 (pmol/L)	13.6	13.3	+0.3	-0.56, +1.2	0.45
TLS Z-score	0.35	0.84	-0.49	-1.2, +0.20	0.16
TH Z-score	0.47	0.75	-0.28	-1.1, +0.5	0.5
Femoral Neck Z-score	0.27	0.59	-0.32	-1.0, +0.4	0.36

The differences between the mean values for each parameter are provided together with their 95% confidence intervals (C.I) and p-values. The minus (-) or plus signs (+) indicate the direction of the difference.

4.6 In-cohort matching to compare TSHR-D727E polymorphism subjects with their controls

Given the interest in the TSHR D727E polymorphism and its proposed influence on BMD (Van der Deure *et al* 2008, Liu *et al* 2012), I evaluated the D727E polymorphism subjects (n = 9) in an identical manner to the TSHR-M carriers (section 4.5).

I selected three control subjects for each D727E subject (excluding D727E subject 132 due to coexisting ATD (TSH; 10.4mU/L, TPO >1300KU/L).

All control subjects had standard TSHR sequence and were matched in the first instance for sex and TPO status. Thereafter controls were matched as closely as possible for age, BMI and TSH (at study attendance) to each D727E subject (raw data displayed in Table 4.15). The comparative analyses on the *matched* data revealed no significant differences between the groups (see Table 4.14).

Table 4.14: Compares the *matched* parameters between D727E subjects and their controls

Parameter	Mean value	Difference	95% C.I	p-value
Age D727E	47.4 years	-2.5	-12.6, +7.6	0.60
Age Control	49.8 years			
BMI D727E	29.2 kg/m^2	0.06	-5.7, +5.8	0.98
BMI Control	29.14 kg/m^2			
TSH D727E	5.12 mU/L	0.2	-1.07, +1.45	0.75
TSH Control	4.93 mU/L			

Differences between matched parameter mean values are provided, together with their 95% confidence intervals (C.I) and p-values. The minus (-) or plus signs (+) indicate the direction of the differences.

Table 4.15: Data on the D727E polymorphism subjects and their controls.

D727E Subjects				Controls			
Study Number	Age (years)	BMI (kg/m^2)	TSH (mU/L)	Study Number	Age (years)	BMI (kg/m^2)	TSH (mU/L)
012	38	25.6	6.68	029	52	22.5	5.44
				134	57	30.3	5.88
				185	57	27.2	5.79
081	63	33.2	4.75	115	57	32.4	6.26
				146	69	31.7	4.07
				185	57	27.2	5.79
124	56	22.3	4.6	027	55	22.7	5.03
				067	62	22.1	4.01
				207	60	21.3	3.5
158	31	22.6	3.26	014	38	25.4	6.6
				077	22	21.9	6.11
				201	49	24.7	3.56
184	47	35.3	3.54	028	53	36.7	4.33
				157	42	35.2	3.04
				192	51	35	4.26
190	46	38.5	4.58	145	47	39.8	5
				157	42	35.2	3.04
				192	51	35	4.26
193	59	33	6.38	080	65	36.6	5.96
				105	53	32.6	3.58
				115	57	32.4	6.26
96	39	23.1	7.16	009	25	20	6.7
				058	42	23.5	4.88
				159	33	27.9	5

Table 4.15. Displays subject; study numbers, ages, BMIs and TSHs for each D727E subjects and their 3 respective controls. Red font represents female subjects and blue font represents male subjects.

The metabolic and bone data on D727E subjects and their controls was compared (Table 4.16). This revealed a lower mean TLS BMD in D727E subjects but no other differences. However, given the low D727E subject numbers contributing to the bone data (n=7 as one subject failed to attend the DXA) the results should be interpreted with *caution*.

Table 4.16: Comparative data on the D727E subjects and their controls.

Parameter	D727E mean	Control mean	Difference	95% C.I	p-value
T4 (pmol/L)	13	13.4	-0.4	-1.9, +1.07	0.6
T3 (pmol/L)	4.34	4.26	+0.08	-0.4, +0.56	0.73
BMD TLS (g/cm^2)	0.92	1.04	-0.12	-0.22, -0.02	**0.025**
BMD TH (g/cm^2)	0.9	1.0	-0.1	-0.25, +0.07	0.22
BMD Femoral Neck (g/cm^2)	0.76	0.84	-0.08	-0.2, +0.04	0.17
TLS T-score	-1.19	-0.08	-1.11	-2.06, -0.15	**0.027**
TLS Z-score	-0.26	0.87	-1.1	-2.1, -0.2	**0.024**
TH T-score	-0.46	0.21	-0.67	-2.0, +0.67	0.27
TH Z-score	0.16	0.86	-0.71	-2.2, +0.75	0.29
Femoral Neck T-score	-0.9	-0.2	-0.7	-1.7, +0.40	0.19
Femoral Neck Z-score	0.0	0.68	-0.68	-1.9, +0.5	0.22
Log^{10} P1NP	1.62	1.52	+0.1	-0.024, +0.22	0.11
Log^{10} CTX	-0.64	-0.7	+0.06	-0.11, +0.2	0.5

Differences between the mean values are provided together with their 95% confidence intervals (C.I) and p-values. The minus (-) or plus signs (+) indicate the direction of the difference.

4.7 Bone parameters, serum calcium, phosphate and alkaline phosphatase evaluated against TFTs

Corrected calcium (calcium), alkaline phosphatase (ALP) and phosphate values are provided on a *bone profile* (a fairly routine blood test on medical inpatients). This was determined on all my study participants.

ALP is an enzyme found in bone and liver primarily. Its levels rise in healing bone (Sharland & Overstall 1979) reflecting accelerated bone formation by osteoblasts, and in liver disorders (including hepatosteatosis; associated with obesity (Webber *et al* 2010)). The ALP provided in a standard *bone profile* does not differentiate organ origin.

Serum calcium and phosphate rise transiently in *acute bone resorption* (reflecting release from hydroxyapatite crystals in bone (Gallacher *et al* 1990)) prior to a prompt homeostatic correction (parathyroid hormone is the major player). Phosphate and, less commonly calcium can be sub-normal in osteomalacia, a complication of profound vitamin D deficiency.

I performed MRAs on each of these parameters to explore for TFT and BMD associations.

4.7.1 Corrected Calcium

The MRA evaluating calcium identified an extremely small positive association with T3 (R: +0.03, p-value 0.009) (see Appendix 6, Table 2).

There were no correlations with:

- BTMs; P1NP or CTX or the ratio of these parameters.
- TSH
- T4
- TPO status
- DXA T or Z- scores (at hip or spine)

The association coefficient (R) between T3 and calcium was explored in a parsimonious stepwise regression analysis (Model 4.5). This showed no diminution in R with the addition of confounders supporting the notion of a positive relationship between calcium and T3.

Model 4.5: Stepwise regression model exploring R between Calcium and T3; assessed singularly and subsequently adjusted for confounders

Step 1; Crude association			
N	**R**	**95%C.I.**	**p-value**
207	+0.026	+0.005, +0.05	0.015
Step 2; Step 1 including male sex			
207	+0.03	+0.05, +0.01	0.004
Step 3; Step 2 including age			
207	+0.03	+0.05, +0.01	0.003
Step 4; Step 3 includes TSH, Waist: hip ratio, activity status and ALP			
206	+0.028	+0.05, +0.007	0.009

Model 4.5 details the number of subjects in the analysis (N), the correlation coefficient (R) between corrected-calcium and T3. The 95% confidence interval (C.I.) and p-values at each step are provided. The minus (-) or plus signs (+) indicate the direction of the association.

4.7.2 Phosphate

The MRA evaluating Phosphate did not reveal associations with thyroid related parameters (T3, TSH, T4 or TPO antibody status). There were also no associations with BTMs or DXA Z or T scores (see Appendix 6, Table 3).

4.7.3 Alkaline Phosphatase

The MRA evaluating ALP showed a very small negative association between ALP and BMD Z-score at TLS and TH (see Appendix 6, Tables 4 and 5).

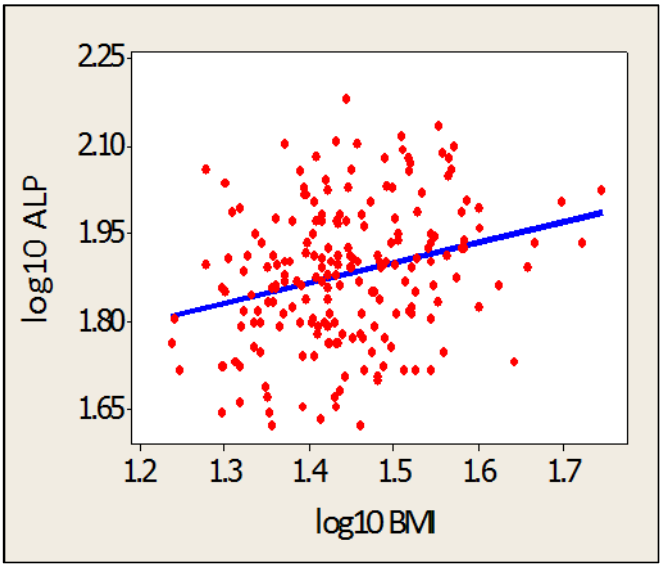
No associations were revealed between ALP and:

- Thyroid parameters; T3, TSH, T4 or TPO status
- BTMs

The lifestyle parameter assessments revealed a positive association between ALP and BMI (see figure 4.12 with associated regression equation), and a negative association between ALP and activity (non-sedentary lifestyle).

This relationship between ALP and BMI is recognised (Webber *et al* 2009).

Figure 4.12: Scatterplot of log^{10} ALP versus Log^{10} BMI



Log^{10}**ALP** = 1.4 + **0.35** Log^{10}**BMI**

P-value < 0.001, R^2 7.5%

Figure 4.12 displays logged BMI on the x-axis and logged ALP on the y-axis. Red dots represent individual's values and the blue line represents the best linear relationship between these parameters.

A parsimonious stepwise regression analysis was performed exploring the relationship between ALP and Z-score at TLS and TH (Model 4.6). This demonstrated that the relationship between ALP and Z-score became apparent when BMI was added to the model (step 2). Given that ALP and BMI are positively correlated (Figure 4.12), and BMI is positively correlated with Z-score (section 4.4.3), the negative relationship between ALP and Z-score is independent of BMI.

Model 4.6: Stepwise regression model exploring the regression coefficient for alkaline phosphatase (Log^{10} ALP) against DXA Z-score at total hip and total lumbar spine; assessed singularly and subsequently adjusted for confounders.

	N	R	95% C.I	p-value
Step 1; crude (adjusting for the respective Z-score site)				
TH;	200	-0.007	-0.03, +0.01	0.48
TLS;	198	-0.01	-0.03, +0.005	0.19
Step 2; Step 1 with adjustment for BMI				
TH;	200	-0.03	-0.05, -0.01	0.002
TLS;	198	-0.02	-0.04, -0.005	0.01
Step 3; Step 2 with adjustment for activity				
TH;	200	-0.03	-0.05, -0.01	0.003
TLS;	198	-0.02	-0.03, -0.003	0.021
Step 4; Step 3 with adjustment for T3, TSH, TPO				
TH;	200	-0.03	-0.05, -0.01	0.004
TLS;	198	-0.02	-0.04, -0.004	0.017

Model 4.6 details the number of subjects in the analysis (N), the correlation coefficient (R) between log^{10}ALP and total hip (TH) Z-score, or log^{10}ALP and total lumbar spine (TLS) Z-score. The 95% confidence interval (C.I.) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

4.8 Analysis of the Bone Turnover Markers

The bone formation marker measured on my study participants was serum P1NP (procollagen type 1 N-terminal propeptide). P1NP represents pro-collagen N-terminal extensions released with the maturation of procollagen to collagen during bone formation (Risteli *et al* 1995).

The bone resorption marker measured on my study cohort was serum CTX (C-terminal telopeptide of type 1 collagen). CTX is a telopeptide fragment of type 1 collagen released during bone resorption (Rosen *et al* 2000).

4.8.1 Bone formation marker; P1NP

The P1NP MRA did not show associations against the predictor variables assessed including:

- Thyroid-related parameters (TSH, T4, T3, TPO status or the thyroid genetic data)
- Lifestyle parameters (BMI or %BF or activity status)
- DXA Z or T-scores (at hip or LS)
- Bone profile parameters (Calcium, ALP or phosphate)

The P1NP MRA is provided in Appendix 6, Table 6

4.8.2 Bone resorption marker; CTX

The CTX MRA did not reveal associations against the predictor variables assessed including:

- Thyroid-related parameters (listed above)
- Lifestyle related parameters (listed above)
- DXA Z or T-scores
- Bone profile parameters (listed above)

The CTX MRA is provided in Appendix 6, Table 7

4.8.3 P1NP CTX product

Enhanced bone loss is associated with a rise in anabolic and catabolic BTMs (Garnero *et al* 2000), whilst a fall in BTMs can signify a response to antiresorptive osteoporotic therapies (Bergmann *et al* 2009). For this reason I have evaluated the 'CTX' 'P1NP' product (log^{10}CTX x log^{10}P1NP).

This P1NP CTX product MRA did not reveal associations with any of the predictor variables (p-values >0.2, R^2 1%) including:

- Thyroid-related parameters (as listed).
- Lifestyle-related parameters (as listed).
- DXA Z or T-scores (at Hip or Lumbar Spine)
- Bone profile parameters

4.8.4 P1NP CTX ratio

I evaluated the P1NP to CTX ratio as I assumed that a more positive ratio would indicate bone formation as the dominant process, whereas a less positive ratio would indicate bone resorption as the prevalent process.

The Log^{10}P1NP/ Log^{10}CTX MRA did not reveal associations with the:

- Thyroid-related parameters (as listed).
- Lifestyle parameters (as listed).

Positive associations with ALP (R +0.5, p-value 0.002, R^2 5%; see Appendix 6, Model 1) and phosphate (R +0.36, p-value 0.02) were seen.

A negative TLS Z-score association of borderline significance was observed but was *not* supported by an associated stepwise parsimonious regression analysis (Appendix 6, Model 2).

4.9 Discussion

The key observations in this chapter are:

(1) Identifying a higher than expected rate of low BMD at the lumbar spine.
(2) Revealing negative DXA Z-score associations with;
    - TSH
    - T3
    - TPO antibody positivity
    - male sex

I will now detail, discuss, and reflect upon the observations made in this chapter.

4.9.1 BMD - TFT observations

I reveal a higher than expected rate of low BMD in this cohort (with 12% having T-scores in the osteoporotic range) despite osteoporosis and evident risk factors for osteoporosis being *exclusion criteria* (section 2.1.2). Exploring this observation further I revealed over-representation of low BMD at the lumbar spine (LS), with BMD discordance affecting 40%. Although discordance itself is not unusual (Woodson 2000, Moayyeri *et al* 2005, El Maghraoui *et al* 2007, Mounach *et al* 2009) the rate of *major discordance* (normal BMD at one site and osteoporosis in another), all with vertebral osteoporosis, appeared to be over-represented in this cohort (occurring in 11% of discordant cases versus a ~3-4% prevalence described (Moayyeri *et al* 2005, Mounach *et al* 2009, Woodson 2000).

When BMD discordance does occur, it is more often in this direction, and is more common in obese subjects (Moayyeri *et al* 2005). However given that obesity can overestimate DXA scores, particularly at the LS (Bansal *et al* 2011), and obese subjects have higher BMDs in general than lean subjects (Albala *et al* 1996, Douchi *et al* 2000, Fawzy *et al* 2011) this does not explain the high rates of low LS BMD in this cohort.

I hypothesise that the prevalence of BMD discordance observed (with lower BMD at LS) *could* relate to differing trabecular to cortical bone ratios between these sites; higher in vertebrae than hip (Clarke 2008, Dempster *et al* 2006). Trabecular and cortical bones differ in many respects including their development (membranous ossification versus endochondral ossification respectively), structure and composition (trabecular bone has a higher surface area to blood ratio), and metabolism; higher in trabecular bone (Clarke 2008); possibly explaining why inflammatory conditions (Akgöl *et al* 2014; Spondyloarthritis, Klaus *et al* 2002; Crohn's disease), chronic diseases (Alcalde Vargas *et al* 2012; liver cirrhosis, Lin *et al* 2012; chronic hepatitis C infection) and acute oestrogen deficiency of early menopause (Elders *et al* 1988, Finkelstein *et al* 2008) has a greater negative effect on trabecular than cancellous bone. As such these bone-types respond differently to stressors, which may include altered thyroid function in SH.

However, given that the ratio of cortical to trabecular bone alters markedly across different regions of a DXA scanned hip (Bohr & Schaadt 1985) other potential explanations for this observation should be sought.

Across the cohort I reveal an inverse relationship between TSH and DXA Z-score at all sites, potentially indicating an adverse TSH effect on bone. My analyses support an independent association between TSH and Z-score at LS, but not at TH. This negative BMD-TSH association specific to LS, could relate to increased TSHR expression at LS, or enhanced TSHR mediated effects at LS; offering alternative theories to the bone-type postulation discussed above.

The negative TSH - BMD relationship observed was not anticipated. In fact, this is contrary to the observations of many studies that associate low TSH (Faber *et al* 1998, Mudde *et al* 1994, Kim *et al* 2015) and low-normal TSH with lower BMD (Kim *et al* 2006); and the inverse for raised or high-normal TSH (Van der Deure *et al* 2008, Chin *et al* 2013)). However, as discussed in the introduction (section 4.1); much uncertainty remains.

The Abe *et al* paper was a landmark paper as it provided *in vivo* and *in vitro* data indicating that TSH had direct effects on bone. The authors found that TSH (via TSHR) had inhibitory effects on osteoclasts, and osteoblasts, and uncoupled the bone remodelling process when TSHR signalling was compromised. However, as effects on osteoclasts were predominant (in TSHR knockdown animals), low BMD was observed (alongside focal osteosclerosis (Abe *et al* 2003)). When I relate these conclusions to my own observations it would suggest that the inhibitory effect of TSHR signalling on osteoblasts outweighs that of osteoclasts, accounting for a negative (rather than positive) association observed between TSH and BMD Z-score at LS. The fact that Abe *et al*'s animals were young mice and my subjects are mostly mature humans may be important in regard to differences in TSHR-bone physiology.

An alternative hypothesis regarding the TSH-Z-score (LS) observation is that TSH may have a negative *indirect* effect on BMD. This would fit in with Abe *et al's* observation of low BMD in TSHR insufficient animals (whose TSH values were raised), mirroring my own observations. The fact that Abe *et al*'s homoinsufficient ($TSHR^{-/-}$) mice continued to have low BMD despite correction of TSH (with thyroxine replacement therapy) cannot be attributed to TSH correction alone as hypersensitivity to thyroid hormones will occur in these animals with congenital hypothyroidism (Bassett *et al* 2008). However, a potential problem with this theory is that it is inconsistent with the observations of Sun *et al* (2008) who describe BMD accrual in response to intermittent exogenous TSH administration, in euthyroid ovariectomised rodents. However, the fact that these rodents were *euthyroid*, and excess TSH exposure was

intermittent may account for its seemingly paradoxical effects. Sun *et al* (2008) suggested that TSH *may* have different effects in different conditions rather like parathyroid hormone (PTH). Indeed, PTH accelerates bone loss in primary and secondary hyperparathyroidism but encourages bone accrual when used therapeutically as an osteoporosis treatment.

A negative relationship between Z-score and T3 was revealed across the cohort (at hip and LS with BMI was the build parameter). This relationship is recognised in hyperthyroidism (Bassett & Williams 2009) but not previously described in an SH cohort. No relationship between BMD and T4 was identified.

A small positive relationship between T3 and TSH (Figure 3.18) was observed across the cohort suggesting that contrary to traditional thinking, T3 levels may increase (rather than decrease) in compensated SH. A negative relationship between TSH and T4 is recognised (Figure 3.17, Hoermann *et al* 2010, Hadlow *et al* 2013). A drop in T4 will activate the D2 deiodinase enzymes to increase T3 production (Gereben *et al* 2008) and there might be overcompensation of this mechanism in SH. If so then altered T3 values (+/- heightened sensitivity to T3) may contribute to enhanced bone loss in SH.

4.9.2 BMD, TPO antibody status and thyroid genetic data

A negative relationship between TPO antibody positivity (+) and DXA Z-score at TLS (R minus 0.65, p-value <0.001) was revealed. This was initially suggested at TH but this was not supported by subsequent analyses.

Associations between inflammatory autoimmune disorders and bone loss are well described (Schett & David 2010), although *not* in autoimmune hypothyroidism or SH. A study evaluating FRAX scores (fracture risk scores) in postmenopausal SH women (82 cases, 51 controls) reported a positive association between TPO+ and FRAX score (Polovina *et al* 2013), as well as between SH and FRAX score (versus euthyroid controls). This study differed from our own in that it reported lower BMD scores affecting the hips of these subjects rather than the spine. Another study evaluating the response of mononuclear cells to a TPO antibody rich serum, reported that this induced synthesis of inflammatory cytokines, including TNF-alpha (Nielsen *et al* 2009); TNF-alpha being a key player in osteoclast mediated bone loss in chronic

inflammation (Azuma *et al* 2000). However, other studies differ in their observations. A Nordic prospective study on adults (>40 years of age) without known thyroid dysfunction or fractures (♀ n = 16,610, ♂ n = 8,595) reported an increased risk of hip fracture (MVHR; 1.87 (95% CI 1.11–3.16) in the 12 year observation period in TPO-ve female subjects, but not TPO +ve. However, TPO status was only assessed on subjects with TSH>4mU/L (included 946 women) (Svare *et al* 2013). Whilst Blum *et al* (2015) did not reval any associations between fracture incidence (n>70,000) and SH.

I performed MRAs to examine the effect of heterozygous TSHR-Ms on bone parameters, and no associations were revealed. However, given my expectation that TSHR function *might* influence BMD (Abe *et al* 2003) and that the mutation (TSHR-M) subject numbers (n=12) were insufficient to reliably exclude differences, further evaluation was undertaken. Although the selected case-control analyses (used for this purpose) initially suggested that TSHR-M subjects *may* be inclined to lower BMD at TLS, this was not subsequently upheld. Another matching analysis was performed to explore potential 'build' differences between the groups and none were suggested. This was assessed because the TSHR is expressed on pleuripotent MSCs and it was hypothesised that altered TSHR activation during development (in TSHR-M subjects) *might* influence final body composition.

My analyses have consistently revealed lower T3s (for an equivalent TSH) in the TSHR-M group. This alteration in T3 would be anticipated to impart a positive BMD effect (given T3 α 1/BMD). However, this wasn't apparent. It is therefore possible that this effect is being offset by a negative BMD effect relating to TSHR-M status (indicating a direct TSHR effect). This hypothesis would then support the work of Terry Davies' group who describe their TSHR-knockout rodents as having lower BMDs than controls (Abe *et al* 2003).

It is important to consider potential *direct* versus *indirect* effects of TSH on BMD. If TSH signalling has a *direct* negative effect on vertebral BMD then TSHR-M carriers should be protected (to some extent) from this effect and would be expected to have higher BMDs than normal sequence SH counterparts (assuming other factors are equivalent). Indeed, the BMD of TSHR-M carriers (with SH) and *euthyroid* normal-sequence controls should be equivalent. This is because reduced TSHR signalling in mutation carriers is assumed to be counter

balanced exactly by a rise in TSH, negating its effect. If instead TSH has an *indirect* negative effect on BMD then TSHR-M status would not influence this process and no BMD differences between SH subjects with or without TSHR-Ms would be expected. As my observations did not reveal BMD differences between these groups, this potentially supports the concept of TSH having an *indirect negative effect* on vertebral BMD. However, given the low TSHR-M numbers in my cohort, my observations (and postulations) may be unreliable. Clearly much larger subject numbers are required to validate these observations.

The analyses performed on the D727E polymorphism subjects versus controls revealed lower TLS DXA-scores in the D727E subjects. If this difference is true, it *is* at a clinically relevant magnitude. However, given the extremely low D727E subject numbers (n=7) this observation could simply be a chance finding. However, this observation supports that of Liu *et al* (2012) who report the polymorphism to be more prevalent in osteoporotic men than their controls (although another study makes an opposing postulation (Van der Deure *et al* 2008)). However, if true the mechanism for this observation is obscure given that the D727E polymorphism appears *not* to be consistently associated with altered TFTs, or altered TSHR constitutive activity.

4.9.3 Non-thyroid related BMD observations across the cohort

Analyses were performed that evaluated the non-thyroid factors that could be affecting BMD. These revealed a positive association between DXA Z-score and WHR (BMI or %BF); activity-status (*non-sedentary* lifestyle), and a negative association with male sex.

The positive relationship between BMD and weight-related parameters or activity are recognised. This relates to the fact that bone is a dynamic organ that adapts to demand; increasing BMD in response to increased transmitted force. A force that may relate to increased weight (Felson *et al* 1993), or to weight-bearing exercise (Todd & Robinson 2003).

Male sex correlated negatively with BMD which was an unexpected finding (Daly *et al* 2013, Rico *et al* 1992). Initially I thought this might relate to a bone protective effect of oestrogen in the pre-menopausal women (Langeland 1978). However, this was not the case as the results were unchanged with the pre-menopausal women removed from the analysis. This

may therefore suggest that SH has a greater negative effect on the BMD of men than women, although the mechanism for this is obscure. Interestingly Lee *et al* (2010) evaluated incident hip fractures in older US adults (≥65 years) and identified a higher rate of fractures in men with SH, but not women.

4.9.4 Analysis of bone profile components and bone turnover markers

Evaluation of bone profile components with the TFT-components and TPO status identified a very small positive association between corrected-calcium and T3, but no ALP or phosphate associations.

An association between calcium and FTHs is recognised in hyperthyroidism (Baxter & Bondy 1966). This is believed to reflect enhanced bone resorption with release of complexed calcium from bone through the action of FTHs (Baran & Braverman 1991). Whether this mechanism is similar in SH subjects cannot be determined (particularly given the absence of vitamin D and parathyroid hormone assessment on these subjects). A relationship between T3 and serum calcium has not been previously described in an SH cohort.

A very small negative association between ALP and Z-score at the hip and TLS was revealed. Given that the ALP-BMI relationship is positive, and the BMI-Z-score relationship is positive, this relationship is independent of BMI. This association has been observed in premenopausal (Krahe *et al* 1997), and postmenopausal women where it is associated with occult spinal fractures (Ross *et al* 2000). Indeed, a fall in bone-specific ALP can indicate a response to bisphosphonate therapy in osteoporosis (Dresner-Pollak *et al* 2000). Given that bone-specific ALP is elevated in healing bone, this was not an unexpected observation.

The analyses assessing the bone turnover markers (BTMs); 'P1NP', CTX', their product, and their ratio did not reveal associations with the TFT-components, TPO+ or DXA Z-scores. Interestingly Polovina *et al* (2013) reported higher FRAX scores in SH women, particularly those with ATD but no differences in their BTMs compared with euthyroid controls.

Although BTMs have been available for some time their use is primarily in research settings (rather than practice). A systematic review (on behalf of the National Institute for Health Research) did not advocate their use as a standard of care for osteoporosis patients (Burch *et*

al 2014) although they *are* considered useful in predicting patient compliance and response to osteoporosis treatments (Bergmann *et al* 2009). Their use as a single point assessment in evaluating cross-cohort trends is limited by wide inter-subject variability (Burch *et al* 2014) possibly explaining the absence of BTM associations in this cohort. Alternatively a change in BMD in SH may be too gradual to be sensitive to these tests, or perhaps its mechanism may not relate to altered bone turnover.

4.9.5 Summary and closing comments

Important BMD associations have been made on this treatment naive, heterogeneous SH cohort in whom I identify a higher than expected rate of low BMD at LS. This suggests that SH *may* have an adverse effect on BMD.

With respect to the TFT components evaluated, I identified a negative association between TSH and Z-score at LS, and a negative association between T3 and Z-score at LS and hip. Thus T3 and TSH may have independent negative effects on BMD in the context of SH. The positive association between TSH and T3 revealed across this cohort was unexpected.

Interestingly, a negative association between BMD and male sex was revealed, indicating that men may be more susceptible to bone loss in SH than women.

In regard to TPO antibody status, a negative association between TPO+ and DXA Z-score at LS was identified, indicating that aetiology of SH *is* likely to be important in regard to its clinical manifestations.

Analyses on the TSHR-M subjects did not reveal associations with either BMD or build parameters. However validation in much larger studies are required.

In conclusion, my observations suggest that bone health should be actively evaluated in SH, particularly in men, people with thyroid autoimmunity or those with additional risk factors for osteoporosis.

The limitations of this study that are of relevance to this chapter are detailed in the closing chapter.

CHAPTER 5: CARDIOMETABOLIC-RISK PARAMETERS IN RELATION TO THYROID FUNCTION AND FOXE1 POLYMORPHISMS IN A SUBCLINICAL HYPOTHYROID COHORT

5.1 Introduction

Thyroid hormones are ubiquitous hormones that act on almost all cells in vertebrates (Eales 1997, Hulbert 2000). They are key regulators of metabolic processes and temperature (Magnus-Levy 1895, Thomas 1957, Tata *et al* 1962). Hyper- and hypothyroidism are associated with an array of changes in metabolic parameters (as well as the bone parameters discussed in Chapter 4).

Hypothyroidism is associated with weight gain (Esmail *et al* 2013), dyslipidaemias (Walton *et al* 1965) (including a rise in LDL-cholesterol and apolipoprotein B (Staub *et al* 1992), diastolic hypertension (Danzi & Klein 2003) and increased systemic vascular resistance (Diekman *et al* 2001). Untreated hypothyroidism is associated with accelerated atherosclerosis (Kocher 1883, Kazi 2003, Cappola & Ladenson 2003) and a predisposition to ischaemic heart disease (Becker 1985) and cerebrovascular diseases (Qureshi *et al* 2006). The physiological and systemic changes of hypothyroidism (reduced cardiac contractility, reduced heart rate and reduced blood volume) reduce exercise capacity. However, congestive cardiac failure attributable to hypothyroidism alone is unusual (Hussain 1997).

The metabolic changes that commonly accompany hyperthyroidism include weight loss, a reduction in lipids (Walton *et al* 1965), (Total Cholesterol (TC), LDL-cholesterol (LDL), ApoB, Lp(a) and HDL-Cholesterol (HDL) (Kung *et al* 1995)), systolic hypertension (Danzi & Klein 2003), reduced systemic vascular resistance (Diekman *et al* 2001), a rise in insulin resistance (Maratou *et al* 2009) and changes in coagulation factors that may predispose to thrombosis (Erem *et al* 2002). The physiological changes of hyperthyroidism (increased myocardial contractility, oxygen demand and vascular volume; widened pulse pressure and increased heart rate) predispose to heart failure (Fadel *et al* 2000). Complications from hyperthyroidism include those that relate to tachyarrhythmias, including atrial fibrillation (AF) with its predisposition to embolic stroke (Jayaprasad & Francis 2005).

It might be assumed, by extrapolation, that adverse cardiometabolic effects would occur but to a lesser extent in subclinical thyroid diseases (ScTDs). In these conditions subjects have normal levels of free thyroid hormones (FTHs) but TSH values outside it. In general, this compensatory change in TSH is sufficient to keep FTH values close to each individual's target value (Andersen *et al* 2003). Consequently, it might be assumed that any metabolic changes observed in the ScTDs may be more attributable to TSH, than to the much smaller relative change in FTH values.

Subclinical hyperthyroidism (Shyper) *has* been associated with an increased risk of tachyarrhythmias, particularly AF (Forfar *et al* 1981), especially in elderly subjects (Cooper 2007) or those pre-disposed to arrhythmias (Haentjens *et al* 2008). SH with TSH ≥10mU/L is associated with adverse metabolic risk parameters (relating to lipids, blood pressure (BP)) (Surks *et al* 2004) and adverse cardiovascular outcomes (Ochs *et al* 2008, Razvi *et al* 2010). However, below this threshold the merit of correcting SH remains unclear (see Table 5.1).

The TSH receptor (TSHR) has been detected in a number of extra-thyroidal sites including fibroblasts and adipose tissue (reviewed in Davies *et al* 2002). Mesenchymal stem cells express TSHR (Bagriacik *et al* 2012) and have the potential to differentiate into different terminal cells (see Figure 1.5) and tissues including fat, muscle, cartilage, mesenchyme and bone; tissues that collectively influence body composition. It has been suggested that TSHR activation might influence lineage specific differentiation (de Lloyd *et al* 2010). Interestingly, an inverse relationship between bone and fat differentiation has been observed (Ng & Duque 2010) which could be influenced by this pathway.

Graves' hyperthyroidism is an autoimmune disease where the TSHR is activated by thyroid stimulating immunoglobulins (TSI). TSI activates thyroidal TSHR, driving thyroid hormone synthesis independently of TSH. Preadipocytes express TSHR and expression is upregulated in fat depots undergoing adipogenesis (Valyasevi *et al* 1999, Crisp *et al* 2000). This observation led to the hypothesis that orbital preadipocyte TSHR may be an important antigen in the pathogenesis of Graves' Orbitopathy (GO) (Crisp *et al* 2000, Ludgate & Baker 2002, Khoo & Bahn 2007). In GO there is pathological accumulation of fat and inflammatory mediators within the confines of the bony orbit resulting in proptosis and ocular injury (Ludgate & Baker 2002). Cell culture experiments exploring the biological effect of TSHR activation on

adipocyte biology indicate that effects are fat-depot source dependent (Zhang L *et al* 2012). These data indicate that TSHR signalling may affect fat cell biology which is itself associated with vascular and metabolic risk (Schäfer & Konstantinides 2011).

The SH subjects recruited to my study all had their BP measured, fasting lipids and glucose assessed, insulin resistance scores (HOMA-IR) calculated and full thyroid function tests (TFTs) performed. In addition, body composition was recorded (by measurement of BMI, waist to hip ratio (WHR), and percentage body fat (%BF). The data was evaluated alongside the TFTs recorded at study attendance.

Participants were all screened for TSHR mutations (TSHR-M), likely to be inactivating mutations in this population. In addition I determined subjects' FOXE1 polyalanine tract length (PTL) polymorphisms (see section 3.1 for more detail). Given that I found TSHR-M status to be negatively associated with T3 (Table 3.9 and 3.10) and the prevalent FOXE1$^{14/14}$ PTL polymorphism to be positively associated with T3 (Model 3.2) it is plausible that these genetic aberrations may influence metabolic parameters. The FOXE1 gene is expressed in a range of cells and tissues with mRNA extracted from liver, pancreas, muscle, brain and fat tissue (Genecards human gene database; FOXE1 (www.genecards.org)). Thus it is conceivable that FOXE1 PTL polymorphisms could influence metabolic parameters.

I have analysed the associations between TFT components and the metabolic data collected on my study participants. These have also been evaluated in association with the genetic data and the TPO antibody status of my subjects. However, as these were not pre-defined primary outcome measures in this study, they are considered as exploratory analyses only.

Table 5.1: Summarises a number of clinical studies to demonstrate the inconsistent metabolic associations reported in Subclinical Hypothyroidism

Parameter	Adverse association with SH	No independent association with SH
Blood pressure	•Cai *et al's* (2011) cross sectional meta-analysis compared SH subjects with controls. Adverse SH associations on MRA with systolic BP (R; +1.89 mmHg, 95% CI; 0.98-2.80, p-value <0.05) and diastolic BP (R; +0.75 mmHg, 95% CI; 0.24-1.27, p-value <0.05) were reported. •Liu et *al's* (2010) cross-sectional questionnaire survey of 1319 adults reported hypertension as more prevalent in SH ♀s than euthyroid ♀s.	•Ye *et al's* (2014) meta-analysis (n=50,147) reported an association between SH and BP that *was* age-dependent. •Walsh *et al* (2006) did not identify an SH-Hypertension association in their cross sectional study (SH; n=105, euthyroid; n=1859). Subjects were not on anti-hypertensives and had no known thyroid disease.
Insulin resistance	•Vyakaranam *et al* (2014) associated SH with insulin resistance (HOMA-IR) in their matched case control study (n=30 pairs). Although SH subjects generally had TSHs >10mU/L (14.2 ±5.23) and non-significantly higher BMIs (27.8 ± 2.8 vs 23.4 ± 1.8). •Maratou *et al* (2009) reported greater insulin resistance and impaired translocation of Glut4 transporters in monocytes of SH subjects (n=12) vs controls (n=21).	•Posadas-Romero *et al* (2014) evaluated insulin resistance and metabolic parameters in a cross sectional study of adult subjects (n=753). An SH - insulin resistance association was identified but dependent on the co-existance of fatty liver.
Lipids	•Bindels *et al's* (1999) cross sectional analysis (n=1200) revealed a positive association between total cholesterol category (<5, 5-8 or >8mmol/L) and prevalence of SH. •Canaris *et al* (2000) reported an adverse association between SH and serum lipids (inclusive of the SH subjects with TSH <10mU/L) from their cross sectional study (n=25,862). •Cheserek *et al* (2014) associated SH with triglycerides in ♂s specifically (R; 0.13, p=0.006), independendent of BMI. Their data was taken from a voluntary health assessment on 1150 university employees.	•Hueston and Pearson (2004) evaluated subjects >40years of age (SH; n= 205, euthyroid; n= 8,013); an association between SH (**TSH; 6.7-14.9mU/L**) and lipids (LDL-cholesterol, HDL-cholesterol, triglycerides) was lost when adjusted for confounding factors. •Vierhapper *et al* (2000) did not correlate SH with LDL-cholesterol or triglycerides (SH; n=1055, euthyroid; n=4886). • Pearce's (2011) review article describes the SH - dyslipidaemia association as *inconsistent* across studies, and an association with HDL-cholesterol as *unlikely.*

Adiposity	•A positive association between adiposity and TSH is described across **euthyroid** cohorts (de Moura Souza & Sichiere 2011, Nyrnes *et al* 2006). • A fall in TSH (from a mean of 4.5 to 1.9mU/L) in response to bariatric surgery induced weight loss (mean drop in BMI; 49 to 32kg/m^2) is described (n=86). SH affected 10.5% of subjects, all of whom normalised post-surgery (Chikunguwo *et al* 2007)	•Garin *et al* (2014) evaluated US subjects ≥65 years of age (SH; n=427, euthyroid; n=2864). No association between SH and weight gain (assessed over 6 years) or adiposity (assessed in a subset of 1276 subjects) was revealed. • A weight loss benefit from treating mild SH (TSH <10mU/L) with TRT over a 6 month observation period was not demonstrated in a blinded, placebo-controlled study (n=40; TRT; n=23, placebo; n=17) (Kong *et al* 2002).
Cardiovascular events	•Ochs *et al's* (2008) meta-analysis associated CV events with SH (**no upper TSH limit**). The RR of CV events in SH was 1.02-1.08 overall (inclusive of only higher quality papers); 1.5 (CI; 1.09-2.09) if <65 years of age, and 1.12 (CI; 1-1.26) if >65 years of age. •Razvi *et al* (2010) evaluated the 20 year follow-up data to the Whickham study (SH; n=97, **SH TSH; 6-15mU/L**; euthyroid n=2279). A greater prevalence of CV disease in SH subjects was reported. However CV events and CV mortality was not different in those with or without TRT, although total mortality was lower in the treated group. •Razvi *et al* (2012) evaluated subjects <70 years of age (n=3093) and >70 years of age (n=1642) with mild SH (TSH; 5-10mU/L). A lower rate of CV events in the younger group with TRT (vs untreated; 4.2% vs 6.6% (CI; 0.39-0.95) but no treatment benefit revealed in the older group.	•Rodondi *et al* (2010) reported an adverse SH-cardiovascular outcome association that applied only to SH subjects with TSH >10mU/L (metanalysis of prospective cohort studies, n=55,287). •Boekholdt *et al* (2010) report on their prospective study (n=11,554 ages; 45-79 years, follow up; 10.6 years). An association between baseline TFTs in the SH range and future CV events or mortality was not seen (a correction for subsequent TRT was not possible). •At this time there is no definitive evidence that treating and correcting mild SH (TSH <10mU/L) has beneficial effects on CV morbidity or mortality (Surks *et al* 2004, Fatourechi 2009).

In Table 5.1 the abbreviations are as follows; CI; Confidence Interval, R; Regression Coefficient, RR; Relative Risk, ♀;Female, ♂;Male, SH; Subclinical Hypothyroidism CV; Cardiovascula, MRA; Multivariate Regression Analysis, vs; versus, n; subject number, TRT; thyroxine replacement therapy to correct SH.

5.2 TPO antibody status and cardiometabolic risk parameters

The metabolic parameters evaluated in this section include BMI (body mass index), WHR (waist to hip ratio), BP (blood pressure), fasting glucose (f-glucose), calculated insulin resistance scores (HOMA-IR), fasting lipid profiles (Total cholesterol (TC), low density lipoprotein cholesterol, high density lipoprotein cholesterol (LDL and HDL respectively), triglycerides (Tg) and self-reported lifestyle factors (activity versus inactivity, smoking history and alcohol consumption). It is recognised that adverse metabolic and lifestyle parameters serve to increase a subject's cardiovascular risk score (Eckel *et al* 2014).

I have evaluated the metabolic data on the cohort according to TPO antibody status (positive (+ve) or negative (-ve)) as shown in table 5.2.

Previous analyses revealed that the TPO +ve group (versus TPO -ve group) were younger (49 versus 54 years), had lower mean BMIs (27.5 versus 29.2 kg/m^2), lower WHRs (0.84 versus 0.88), were less sedentary (40% versus 57%) and the group comprised more women (84% versus 67%). In addition, they had higher TSHs (5.8mmol/L versus 4.8mmol/L), and lower T4s (12.7mmol/L vs 13.6mmol/L) (see Table 4.4).

Table 5.2 reveals that, in addition, the TPO +ve group have less smoking history, fewer hypertensive subjects, and borderline higher mean HDLs. Thus it appears that the TPO -ve subjects display higher cardiovascular risk scores on account of their lifestyle parameters. The next section will explore for *independent* TPO antibody - metabolic parameter associations.

Table 5.2: Cardiometabolic data in relation to TPO antibody status

Parameter	TPO +ve (n=104)	TPO -ve (n=104)	Difference	95% C.I	p-value
Number with >10 pack year smoking history	24	40	-15%	-0.28 to -0.03	**0.015**
Number of 'heavy' alcohol consumers	7	8	+1%	-0.06 to +0.08	0.79
Number with Hypertension	23	40	-16%	-0.29 to -0.04	**0.009**
Number with Diabetes	7	13	-6%	-0.14 to +0.02	0.16
Number with IHD or CVD	4	9	-5%	-0.11 to +0.02	0.15
$\bar{x}$ TC (mmol/L)	5.36	5.22	0.14	-0.2 to +0.45	0.41
$\bar{x}$ LDL (mmol/L)	3.31	3.21	+0.1	-0.18 to +0.38	0.5
$\bar{x}$ HDL (mmol/L)	1.42	1.32	+0.1	-0.002, +0.2	**0.05**
$\bar{x}$ Tg (mmol/L)	1.48	1.56	-0.08	-0.5 to +0.34	0.71

The mean ($\bar{x}$) % differences between the groups are provided with confidence intervals (C.I) and p-values. Minus (-) or plus (+) signs indicate the direction of the differences. Heavy alcohol consumption was considered as >14 units/week (female) and >21 units/week (male). Abbreviations include IHD; Ischaemic Heart Disease and CVD; Cerebrovascular Disease.

5.3 Cardiometabolic-risk parameters in relation to TFTs, TPO status and thyroid genetic data

A diagnosis of hypertension is made when repeated BP values are elevated. A systolic BP >140mmHg and/or diastolic BP >90mmHg is generally used to define hypertension (Mancia *et al* (2013) on behalf of the European Society of Hypertension, and Cardiology). Untreated hypertension causes vascular damage through a range of mechanisms that include endothelial injury, endothelial dysfunction, end arteriolar infarction, oxidative stress and acceleration of the atherosclerotic process. These processes collectively contribute to end organ injury (Alexander 1995).

The BP associations across the cohort are provided in section 5.3.1-5.3.3.

In my multiple regression analyses (MRAs) the effect of the affirmative binary parameter on the dependent variable is being assessed. These were as follows:

- Activity(1) predicts the effect of a *non-sedentary* lifestyle
- FOXE1(14) predicts the effect of homozygosity for the FOXE1-14 PTL polymorphism
- TPO(+ve) predicts the effect of TPO antibody positivity
- Alcohol(1) predicts the effect of consuming alcohol (as opposed to tee-total).
- Smoking(1) predicts the effect of having a >10 pack year history
- TSHR-M predicts the effect of carrying a TSHR mutation
- Male(1) predicts the effect of male sex

For detail regarding the interpretation of MRAs please see Chapter 2, section 2.19.

The values used in the BP analyses represent the mean of 3 readings taken at study attendance (see section 2.4). All participants were fasted and were advised to take their antihypertensive medication *after* their clinical assessment.

The first BP MRA I performed evaluated against a range of parameters that included T4, smoking history, alcohol history, TSHR-M and TSHR polymorphism (D727E and P52T) status. However, as these parameters *did not* associate with BP (and are not known or expected to influence BP), for simplicity (and to reduce the likelihood of type 1 errors) they are *not* included in the BP MRAs that follow.

5.3.1 Systolic Blood Pressure

This MRA explored the relationship between systolic BP and WHR, activity status, male sex, TSH, T3, TPO+, subject age, and FOXE1 PTL status; see MRA below and its corresponding table (table 5.3).

Systolic BP = 49 + 18**WHR** - 1.4**Activity**(1) + 2.5**Male**(1) - 1.5 Log^{10}**TSH** + 11.7**T3** + 0.6 - 0.9**TPO+ve**(1) + 0.4**Age** - 7.4**FOXE1(14)**

N=201 R^2 = 31%

Table 5.3: MRA evaluating systolic BP against the predictors listed

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+49	14.6	+21, +78	0.01
WHR	+19	15	-10, +48	0.2
Activity(1)	-1.4	2.2	-5.7, +3	0.53
Male(1)	+2.6	3	-3, +8.4	0.39
Log^{10} TSH (mU/L)	-1.5	5.8	-12, +10	0.8
T3 (pmol/L)	+11.7	2.1	+7.5, +16	<0.001
TPO+ve(1)	-0.9	2.2	-12, +10	0.7
Age (years)	+0.4	0.09	+0.3, +0.6	<0.001
FOXE1(14)	-7.4	2.2	-11.6, -3.2	0.001

Table 5.3 provides the regression coefficient (R) for each predictor assessed in the MRA evaluating Systolic BP. The corresponding standard errors (SE), confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

The systolic BP MRA reveals the following associations:

- A positive T3 association (R +11, p-value <0.001)
- A negative FOXE1(14) association (R -8, p-value <0.001).
- A positive association with age (R +0.4, p-value <0.001), as expected.

When this analysis was repeated substituting BMI for WHR the associations were similar but the significance scores improved (see Appendix 7, Table 1).

5.3.2 Diastolic Blood Pressure

The MRA evaluating diastolic BP (equivalent to the systolic BP above) failed to reveal associations against the predictor variables (including T3 [R+0.02, p-value 0.55], and FOXE1(14) [R-0.06, p-value 0.17]).

However, when BMI was substituted for WHR the associations changed; the T3 association fell *just* short of statistical significance, and the FOXE1(14) association was restored (R; minus 3.5, C.I; minus 6.3 to minus 0.7, p-value 0.015). See Appendix 7, Table 2.

5.3.3 Exploring the relationship between blood pressure and T3, and blood pressure and *FOXE1* PTL polymorphism status

The blood pressure regression analyses thus far indicate that T3 and *FOXE1* PTL polymorphism status correlate with BP. To explore these associations further I used stepwise parsimonious regression models.

Model 5.1 explores the relationship between BP and T3 whilst Model 5.2 explores the relationship between BP and FOXE1(14) PTL polymorphism status.

BMI is the build parameter used in these regression models.

Model 5.1 reveals a positive *crude* association between systolic BP, and diastolic BP with T3 (Step 1). The coefficient for both values is notable (~3 for diastolic and ~11 for systolic) although ~3 fold higher for systolic than diastolic BP.

Model 5.1. Stepwise regression model exploring the association between blood pressure and T3; assessed singularly and subsequently adjusted for confounders.

		Systolic				**Diastolic**	
N	**R**	**95%C.I.**	**p-value**	**N**	**R**	**95%C.I.**	**p-value**
Step 1; crude (against T3 only)							
207	+11	(+6.6, +15.4)	<0.001	206	+3.8	(+1, +6.5)	0.008
Step 2; Step 1 also adjusted for BMI (log^{10}BMI)							
206	+9.6	(+5.2, +14)	<0.001	206	+3.1	(+0.4, +5.8)	0.037
Step 3; Step 2 also adjusted for FOXE1 PTL(14) polymorphism status							
201	+11	(+6.7, +15.3)	<0.001	201	+3.8	(+1, +6.6)	0.008
Step 4; Step 3 also adjusted for age and sex							
201	+10.8	(+6.7, +15)	<0.001	201	+3	(+0.3, +5.7)	0.035
Step 5; Step 4 also adjusted for TPO positivity, activity and TSH (log^{10} TSH)							
201	+10.8	(+6.6, +15)	<0.001	201	+2.7	(-0.1, +5.5)	0.058

Model 5.1 details the number of subjects in each analysis (N), the correlation coefficient (R) between systolic BP and T3, or diastolic BP and T3. Alongside is the 95% confidence interval (C.I) for each analysis step and its p-value. The minus (-) or plus sign (+) indicate the direction of the association.

The statistical significance of the association (p-value) falls for diastolic BP as confounders are added in to the analysis (particularly at the final step), but remains highly significant for systolic BP. This could indicate that the association with T3 applies only to systolic BP, or alternatively that the sample size is underpowered to consistently reveal this relationship with diastolic BP.

Model 5.2 explores the relationship between BP and FOXE1 $PTL^{14/14}$ polymorphism status. Although the crude associations fall short of statistical significance for systolic and diastolic readings, they become significant when T3 is added into the model at step 2 and R rises with the addition of confounding factors. This relationship is more substantial for systolic than diastolic BP in regard magnitude of R (-7.2, versus -3.3), and its p-value (<0.001 versus 0.014).

Model 5.2: Stepwise regression model exploring the association between blood pressure and FOXE1$^{14/14}$ polymorphism status; assessed singularly and subsequently adjusted for confounders

	Systolic BP				Diastolic BP		
N	**R**	**95% C.I.**	**p-value**	**N**	**R**	**95% C.I.**	**p-value**
Step 1; crude association							
202	-4.7	(0.12, -9.5)	0.058	202	-2.7	(-5.6, +0.2)	0.07
Step 2; adjusted for Model 1 including T3							
202	-6.8	(-2.2, -11.4)	0.004	202	-3.5	(-6.4, -0.6)	0.02
Step 3; adjusted for model 2 including BMI (log^{10}BMI)							
201	-7	(-10.9, -2.5)	0.003	201	-3.5	(-6.4, -0.6)	0.016
Step 4; adjusted for model 3 including age and male sex							
201	-7.6	(-12, -3.7)	<0.001	201	-3.5	(-6.2, -0.8)	0.014
Step 5; adjusted for model 4 including TPO positivity, activity and TSH (log10 TSH)							
201	-7.8	(-12, -3.6)	<0.001	201	-3.5	(-6.3, -0.7)	0.014

Model 5.2 details the number of subjects in each analysis (N) and the correlation coefficient (R) between either systolic BP or diastolic BP and the FOXE1 $PTL^{14/14}$ polymorphism. The corresponding 95% confidence intervals (C.I) and p-values at each step are provided. The minus (-) or plus signs (+) indicate the direction of the association.

To summarise, Model 5.1 supports the hypothesis of an independent and positive relationship between T3 and systolic BP. Model 5.2 reveals a negative association between FOXE1$^{14/14}$ PTL polymorphism status and BP.

5.4 Adiposity

Body Mass Index (BMI) is a calculated value used to indicate a subject's weight category. For example, a BMI <18.5 suggests a subject is underweight, whereas a value >40 indicates 'morbid obesity' (WHO categories applicable to Caucasians).

However, the reliability of BMI for accurate weight categorisation is limited by the fact that it does not consider the subject's build. For example, an athlete with high muscle mass may be categorised incorrectly as obese, whilst a subject with isolated central obesity and thin limbs or low muscle mass (particularly the elderly) will have their obesity category underestimated (Rothman 2008). Similarly, tall people tend to have their adiposity underestimated (versus short people) (Bagust & Walley 2000). However, in the majority of cases BMI is a helpful indicator of weight category.

Percentage body fat (%BF) is another way of measuring adiposity and this was measured in all my study participants. However, this value has been suggested to overestimate obesity in normal BMI subjects, particularly those of Asian origin. The counter view is that BMI is less accurate than %BF in these groups (Carpenter *et al* 2013).

Another popular tool to categorise adiposity is the waist to hip ratio (WHR) or even waist circumference alone. These measures of *central* obesity are simple to perform and helpful adverse cardiovascular risk predictors (de Koning *et al* 2007). They have been suggested by some to be more accurate adverse risk predictors than %BF or BMI (Cepeda-Valery *et al* 2011).

An MRA to explore the predictors that might influence, or associate with BMI is shown below. The TPO antibody and genetic data are not included in this analysis as no associations against these predictors were revealed (R +/- 0.02, p-values >0.2).

BMI was log transformed prior to MRA to normalise the data. BMI was evaluated against smoking, alcohol and activity status, male gender, TSH, T3 and T4 values (as shown below). The relationship between these predictors and BMI is summarised in table 5.4.

Log^{10}**BMI** = 1.35 + 0.03**Smoking**(1) - 0.01**Alcohol**(1) - 0.06**Activity**(1) + 0.02**Male**(1) - 0.04 **l**og^{10}**TSH** + 0.02**T3** + 0.004**T4**

N=207 R^2 = 18%

Table 5.4: MRA evaluating BMI against the predictors listed

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+1.4	0.07	+1.3, +1.5	<0.001
Smoking(1)	+0.03	0.01	+0.01, +0.05	0.02
Alcohol(1)	-0.01	0.02	-0.05, +0.03	0.7
Activity(1)	-0.06	0.01	-0.08, -0.04	<0.001
Male(1)	+0.016	0.014	-0.01, +0.04	0.3
Log^{10} TSH (mU/L)	-0.04	0.04	-0.12, +0.04	0.2
T3 (pmol/L)	+0.02	0.01	-3×10^{-3}, 0.05	0.09
T4 (pmol/L)	+0.004	0.004	-0.04, +0.12	0.4

Table 5.4 provides the regression coefficient (R) for each predictor assessed in the MRA evaluating BMI. The corresponding standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus sign (+) indicate the direction of the association.

The BMI MRA did not reveal associations with the TFT components (or TSHR-M or TPO antibody status (data not shown)).

In regard to the lifestyle factors associated with BMI; activity was negatively associated, and smoking status was positively associated, although both correlation coefficients were small.

When I compared the MRA results substituting %BF for BMI, the results were similar although male sex showed a negative correlation (see Appendix 7, Table 3). When this was performed using WHR (in place of BMI) a positive association with male sex was seen (see Appendix 7, Table 4). These associations reflect the recognised build differences between men and women (Stevens *et al* 2010).

5.5 Fasting Glucose

The study subjects excluded from this analysis were diabetics treated with insulin (n=5) or on oral hypoglycaemic agents (n=3). In addition I excluded one subject with probable newly diagnosed diabetes (fasting glucose (f-glucose) 11mmol/L (Diabetes ≥7mmol/L).

The MRA to evaluate the f-glucose associations (data not shown) did not demonstrate correlations with TFT components, TPO status or the thyroid genetic parameters assessed (Rs <0.02, p-values >0.16). As expected, f-glucose correlated positively with BMI (or WHR or %BF when these parameters were substituted into the equation).

5.6 Insulin resistance: HOMA-IR

HOMA-IR is a non-invasive method to estimate *insulin resistance* using fasting insulin and glucose values. It is commonly used in clinical research settings. The calculation for HOMA-IR is shown below;

HOMA-IR = glucose (mmol/L) x Insulin (mU/L)/ 22.5

HOMA-IR was log transformed prior to MRA evaluation to normalise the data.

The HOMA-IR MRA was initially evaluated against TPO positivity, FOXE1(14) status, TSHR-M and polymorphism status (D727E & P52T). However, as these parameters did not associate with HOMA-IR to any extent (Rs <0.007, p-values >0.3), for simplicity (and to avoid type 1 errors) they are not included in the MRA below. Similarly, alcohol intake and smoking status showed no associations and are not included.

HOMA-IR was evaluated against BMI, activity status, male gender, TSH, T3 and T4 values (as shown below). The relationship between these predictors and HOMA-IR is provided in table 5.5.

Log^{10} **HOMA-IR** = -3.5 + 2.3Log^{10}**BMI** - 0.2**Activity**(1) + 0.1**Male**(1) - 0.02Log^{10} **TSH** + 0.1**T3** - $3X10^{-3}$**T4**

N = 196, R^2 = 38%

Table 5.5: MRA evaluating HOMA-IR against the predictors listed

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-3.5	0.5	-2.5, -4.5	<0.001
Log^{10}BMI	+2.3	0.3	+1.7, +2.9	<0.001
Activity(1)	-0.2	0.05	-0.1, -0.3	<0.001
Male(1)	+0.1	0.06	-0.02, +0.2	0.05
Log^{10} TSH (mU/L)	-0.02	0.15	-0.3, +0.3	0.9
T3 (pmol/L)	+0.1	0.06	-0.02, +0.2	**0.05**
T4 (pmol/L)	$-3x10^{-3}$	0.02	-0.04, +0.04	0.9

Table 5.5 provides the regression coefficients (R), standard errors (SE), 95% confidence interval (C.I) and p-values for each predictor evaluated in the HOMA-IR MRA. The minus (-) or plus signs (+) indicate the direction of the association.

The HOMA-IR MRA suggests T3 as the only thyroid parameter that *may* correlate although its positive association is small, and p-value of borderline significance (R +0.1, p-value 0.05).

In regard to the lifestyle and personal characteristics evaluated against HOMA-IR, BMI associates positively, and activity associates negatively (both are as expected).

When WHR was substituted in place of BMI (with T4 removed as it showed no association) the correlations were similar although the association with T3 was now significant (R +0.2, p-value 0.006 (See Appendix 7, Table 5).

The relationship between T3 and HOMA-IR was explored in a parsimonious stepwise regression analysis shown in Model 5.3. This supports the observation that T3 correlates positively with HOMA-IR.

Model 5.3: Stepwise regression model exploring the association between HOMA-IR and T3; assessed singularly and subsequently adjusted for confounders.

N	R	95%C.I.	p-value
Step 1; crude (against T3 only)			
199	+0.2	(+0.06, +0.3)	0.003
Step 2; Step 1 adjusted for WHR			
197	+0.12	(+0.02, +0.2)	0.03
Step 3; Step 2 adjusted for activity			
197	+0.13	(+0.03, +0.2)	0.016
Step 4; Step 3 adjusted for male sex			
197	+0.15	(+0.04, +0.2)	0.01
Step 5; Step 4 adjusted for TSH			
197	+0.14	(+0.04, +0.2)	0.007
Step 6; Step 5 adjusted for TPO, TSHR-M, FOXE1, smoking & alcohol status			
192	+0.17	(+0.05, +0.29)	0.003

Model 5.3 details the number of subjects in the analysis (N), the correlation coefficient (R) between Log^{10}HOMA-IR and T3, its associated 95% C.I and p-value for each step. The minus (-) or plus sign (+) indicate the direction of the association.

5.7 Lipids

In regard to cardiovascular risk parameters (generally applicable to higher risk individuals), an elevated LDL cholesterol is considered an adverse risk predictor (reflecting transfer of cholesterol to the body), whilst HDL cholesterol is considered protective (reverse transports cholesterol back to the liver).

The Total Cholesterol (TC) to HDL cholesterol ratio (TC/ HDL) is commonly reported in a lipid profile. An adverse ratio (variably considered >4.5) is another adverse risk predictor that is considered to be a better risk predictor than individual lipid components (Millán *et al* 2009).

Triglycerides (Tg) are associated with obesity, the metabolic syndrome, and are inversely associated with HDL. However, their independent influence on cardiovascular outcomes remains unclear (Miller *et al* 2011).

5.7.1 LDL Cholesterol

The MRA evaluating LDL did not show associations with TFT components, TPO status or the thyroid genetic data (TSHR-M or polymorphism status or FOXE1(14) status). In addition, lifestyle factors (activity status or any of my measure of adiposity) did not show associations. The LDL MRA with its corresponding table are provided in Appendix 7, Table 6.

5.7.2 HDL Cholesterol

The MRA evaluating HDL cholesterol did not show associations with; TFT components, TPO status or the genetic data.

The associations revealed were as follows:

- A negative association with BMI (R minus 0.1, p-value 0.008)
- A positive association with activity (R +0.03, p-value 0.001)
- A negative association with male sex (R minus 0.05, p-value <0.001).

The MRA for this analysis is provided in Appendix 7, Table 7.

5.7.3 Total Cholesterol to HDL ratio

The MRA evaluating the TC: HDL did not show associations with the TFT components, TPO antibody status or the genetic data.

The personal and lifestyle factors that showed a positive association were:

- BMI (R +0.35, p-value< 0.001)
- Male sex (R +0.06, p-value 0.005)

The MRA for this analysis is provided in Appendix 7 Table 8.

5.7.4 Triglycerides

The triglyceride (Tg) MRA was initially evaluated against TPO antibody status, the genetic data, alcohol and smoking status. However, as these parameters did not associate with Tg to any extent they are not included in the MRA below.

The Tg MRA was evaluated against activity status, male gender, BMI, T3, TSH, and T4 as shown below. The relationship between these predictors and Tg is provided in Table 5.6.

$$\text{Log}^{10}\textbf{Tg} = -1 - 0.06\textbf{Activity}(1) + 0.04\ \textbf{Male}(1) + 0.6\ \text{Log}^{10}\textbf{BMI} + 0.08\textbf{T3} + 0.003\ \text{Log}^{10}\textbf{TSH} - 2\text{x}10^{-3}\ \textbf{T4}$$

$$N=204 \qquad R^2 = 17\%$$

Table 5.6: MRA evaluating triglycerides against the predictors listed

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-1	0.3	-1.6, -0.4,	<0.001
Activity(1)	-0.06	0.03	-0.12, -1x10^{-3}	0.04
Male(1)	+0.04	0.03	-0.02, +0.1	0.3
Log^{10}BMI	+0.6	0.2	+0.2, +1	0.001
T3 (pmol/L)	+0.08	0.03	+0.02, +0.14	**0.01**
Log^{10}TSH (mU/L)	-3x10^{-3}	0.09	-0.17, +0.18	1.0
T4 (pmol/L)	-2x10^{-3}	0.01	-0.02, +0.02	0.9

Table 5.6 provides the regression coefficients (R), standard errors (SE), 95% confidence intervals (C.I) and p-values for each predictor variable evaluated in the Tg MRA. The minus (-) or plus sign (+) indicate the direction of the association.

The Tg MRA revealed the following associations:

- A small positive association with T3 (R +0.08, p-value 0.01); this was the only thyroid parameter to show an association.
- Of the lifestyle and personal characteristics, BMI correlated positively (R +0.6, p-value 0.001) and activity correlated negatively (R-0.06, p-value 0.04) as expected.

I subsequently evaluated the relationship between T3 and Tg further using a stepwise parsimonious regression model (Model 5.4).

Model 5.4: Stepwise regression model exploring the relationship between triglycerides and T3; assessed singularly and subsequently adjusted for confounders

N	R	95% C.I	p-value
Step 1; crude (against T3 only)			
205	+0.11	(+0.05, +0.17)	<0.001
Step 2; step 1 adjusted for Log^{10}**BMI**			
204	+0.09	(+0.03, +0.15)	0.003
Step 3; step 2 adjusted for male sex			
204	+0.08	(+0.02, +0.14)	0.005
Step 4; step 3 adjusted for Log^{10}**TSH and T4**			
204	+0.08	(+0.02, +0.15)	0.01

Model 5.4 details the number of subjects in the analysis (N), the regression coefficient between log^{10}*triglyceride and T3, its associated 95% confidence interval (C.I) and p-value for each step. The plus sign (+) and minus (-) signs indicates the direction of the association.*

5.8 Discussion

This chapter explored the association between metabolic parameters (measured in my SH cohort) and TFT components, the genetic characteristics of subjects (TSHR-M status, TSHR polymorphism status, and FOXE1 PTL polymorphism status) and their TPO antibody status. These analyses identified a number of interesting associations.

The evaluations on blood pressure (BP) as the outcome variable revealed a positive association between *systolic* BP and T3, but no relationships with TSH or T4. This is consistent with the recognised association between hyperthyroidism and systolic hypertension (Prisant *et al* 2006), thought to be T3-mediated. However, this relationship has not been previously described in an SH cohort. This observation suggests that any tendency to hypertension in SH subjects (inconsistently described in some studies (Cai *et al* 2011)) *may* be T3-driven. The absence of an association between TSH and BP (even after removing T4 and then T3 from the analysis) suggests that this is not related to TSH (despite the weak positive association between T3 and TSH revealed across the cohort; Figure 3.18).

Given the positive association between TSH and T3 (and an inverse association between T4 and T3) across this cohort, I hypothesise that thyroxine initiation to correct SH *might* also reduce T3. Remarkably, the effect of thyroxine initiation (in SH) on T3 levels (though described to be within population norms) has not been studied. In fact, monitoring of T3 in these subjects is not advised (American Association of Clinical Endocrinologists and American Thyroid Association Taskforce on Hypothyroidism in Adults 2012 (Garber *et al* 2012)). It is recognised that a falling T4 (in progressive SH) upregulates T4 to T3 conversion peripherally (Lum *et al* 1984) which might explain this observation. If true, one would anticipate a potential decline in BP (dependent on degree of SH, and a subject's tendency to hypertension) as SH is corrected with thyroxine, and a potential adverse BP effect related to treating hypothyroidism or SH with T3. However, where T3 replacement therapy has been used (+/- T4) and evaluated in healthy hypothyroid subjects (in extremely small numbers and over short time periods) an adverse BP effect was not reported (Bunevicius *et al* 1999).

My analyses revealed a surprising negative association between BP and the homozygous *FOXE1* PTL 14 polymorphism (versus other PTL combinations). I previously identified that this genotype was associated with higher T3 values for an equivalent TSH (Model 3.2). If this was simply T3-mediated I would predict a higher and not a lower BP in this group. When I review stepwise regression model 5.2 it is apparent that the negative association with *FOXE1*(14) and BP becomes more apparent when T3 is added into the model. Given that the homozygous 14 PTL genotype is the prevalent and normal genotype, it is rather the case that alternative genotypes have lower T3s (relative to TSH) and may be less tolerant to the systemic vascular effects of T3 (as reflected in BP). It would therefore be interesting to explore *FOXE1* PTL polymorphism frequencies according to tolerance (or otherwise) of the systemic effects of hyperthyroidism, and also to explore their prevalences in matched hypertensive versus normotensive cohorts.

The *FOXE1* gene plays a vital role in thyroid morphogenesis and influences the transcription of thyroglobulin and thyroid peroxidase (Francis-Lang *et al* 1992). Bullock *et al* (2012) reported higher transcriptional activity of the *FOXE1* 14 PTL polymorphism versus the 16, potentially explaining functional differences between these common polymorphisms. The *FOXE1* gene is also expressed in a number of extra-thyroidal tissues that include the central nervous system and liver (Genecards human gene database; FOXE1 (www.genecards.org)). It

is therefore feasible that subtly altered FOXE1 function in either or both these sites could also be behind this observation.

Comparative observations evaluating cardiovascular risk parameters according to TPO antibody status indicated a higher score amongst TPO negative (versus positive) subjects (Table 5.2). However, subsequent MRAs indicate that this difference was attributable to differing characteristics of the groups (comorbidities, lifestyle and personal characteristics and) and did not relate to TPO status *per se*.

My analyses did not identify associations between TFT parameters and any of the measures of adiposity used in this study (BMI, WHR or %BF). Therefore my observations do not support an association between adiposity and TSH that has been suggested in some studies (though these associations tend to have been reported across euthyroid cohorts (Mehran *et al* 2012, Nyrnes *et al* 2006)).

The fasting glucose MRAs revealed no associations with the thyroid parameters assessed. However, the HOMA-IR analysis showed a small positive association with T3 (Model 5.3). An association between insulin resistance and T3 is recognised (Bakker *et al* 1999) though appreciated in the context of hyperthyroidism (Chu *et al* 2011). This association between T3 and HOMA-IR has not been previously observed within an SH cohort. No association between HOMA-IR and TSH was revealed.

My analyses evaluating the lipid profile (TC, LDL and HDL cholesterol values) did not reveal associations with the TFT components or the genetic parameters evaluated in this study. This would support the notion that mild SH (TSH <10mU/L) does not influence cholesterol values appreciably (Vierhapper *et al* 2000, Hueston & Pearson 2004). However, this remains an area of contention (Pearce 2012).

The triglyceride evaluations revealed a small unexpected positive association with T3, but no associations with T4, TSH or the genetic parameters evaluated. I found this association described in one previous study that evaluated elderly subjects with no history of thyroid diseases (Maugeri *et al* 1999). A rise in triglycerides has been described in hyperthyroid and hypothyroid subjects relative to euthyroid controls (Nikkilä & Kekki 1972). This positive T3-triglyceride association has not been previously described in an SH cohort.

In conclusion, my analyses revealed a positive association between T3 and systolic BP, HOMA-IR, and triglycerides across this SH cohort. My genetic analyses identified an unexpected negative association between the FOXE1$^{14/14}$ polymorphism and blood pressure which warrants further investigation. TPO antibody status, TSH and T4 values did not associate with any of the metabolic parameters assessed in this cohort and are therefore unlikely to have clinically significant independent metabolic (non-bone) effects in this context.

These findings would suggest that alterations in T3 or T3 sensitivity may be behind the adverse metabolic parameters reported, albeit inconsistently, in the context of SH. There were no independent metabolic parameter - TSH associations demonstrated in this study.

The limitations of this study that are of relevance to this chapter are detailed in the closing chapter.

CHAPTER 6: STUDY OVERVIEW AND CONCLUSIONS

Subclinical Hypothyroidism (SH) has remained a popular subject for research since its definition and description in 1973 (Hall & Evered 1973). The clinical management recommendations for hyper and hypothyroidism advocate its treatment and correction in *all* cases, whereas the recommendations relating to the subclinical thyroid diseases (ScTD) are much less specific. Recent guidelines advice treating and correcting persistent SH routinely when the TSH exceeds 10mU/L in subjects less than 65 years of age (Pearce *et al* 2013; ETA Guidelines). This is primarily to avoid future morbidity related to progression of SH to hypothyroidism though there *may* also be metabolic benefits on lipids (Caraccio *et al* 2002, Razvi *et al* 2007) and endothelial function (Cabral *et al* 2011) from correcting SH. The merit of treating SH at lower TSH thresholds remains uncertain. These guidelines also advocate a trial of thyroxine replacement therapy (TRT) in subjects symptomatic of SH (despite TSH <10mU/L) with continuation of TRT in those describing symptomatic benefit (Pearce *et al* 2013).

There is ample data linking suppressed TSH in subclinical hyperthyroidism (Shyper) to accelerated bone loss (Faber *et al* 1998, Mudde *et al* 1994), particularly in post-menopausal women. Specialty guidelines support this statement by advocating the treatment and correction of Shyper in subjects' ≥65 years of age and those with osteoporosis (American Thyroid Association & American Association of Clinical Endocrinologists in Bahn *et al* 2011).

As reiterated throughout this thesis; the bone health and metabolism data in SH is inconsistent and even contradictory across studies. However, most studies suggest beneficial bone mineral density (BMD) associations relating to a rise in TSH (the inverse of Shyper). The specialty guidelines reflect this uncertainty by refraining from making bone related recommendations regarding the clinical management of SH.

SH is often considered to be a uniform condition which as explained throughout this thesis is not the case. It may be that its clinical implications are in fact cause specific. It is recognised for example that the rate of progression from SH to hypothyroidism (though not inevitable) is higher in subjects with thyroid autoimmunity (Vanderpump *et al* 1995). However, in

general, the clinical guidelines do not differentiate cause of SH when recommending on its management.

The landmark paper by Abe *et al* (2003) was instrumental in my decision to explore bone parameters (BMD and bone turnover markers (BTMs)) in treatment naïve SH subjects and to relate this to aetiology (heterozygous TSHR mutations (TSH-M) versus normal sequence subjects, and thyroid autoimmunity versus other causes). The Abe *et al* group linked accelerated bone loss and low BMD in general (alongside focal areas of osteosclerosis) with TSH receptor (TSHR) insufficiency in their genetically modified mice. This was in spite of normal free thyroid hormone values (FTHs) in animals heterozygous for TSHR insufficiency (with SH) or normalisation of FTHs (through TRT) in animals lacking TSHR (who are otherwise hypothyroid). This group described TSH as *a negative regulator of bone remodelling* with inhibitory effects demonstrated on osteoblasts and osteoclasts. They believed that their observations indicated a direct role for TSHR signalling in bone.

I assumed that by evaluating bone-related parameters in human subjects heterozygous for inactivating TSHR-Ms (genetically similar to heterozygous TSHR knockdown mice) this might add helpful human observational data to this work.

On planning this project I anticipated that subjects heterozygous for inactivated TSHR-Ms would reside predominantly in SH cohorts (rather than euthyroid or hypothyroid cohorts (Cassio *et al* 2013). I also anticipated that subjects bearing TSHR-Ms would be seronegative for thyroid autoantibodies (in the main), as the mechanism for their SH is not autoimmunity (Alberti *et al* 2002). As such I set out to recruit a cohort of treatment naïve SH subjects with primary endogenous SH, and to screen all participants for TSHR-Ms (expected to be *inactivating* mutations in this context). Treatment naive subjects with primary SH were recruited to avoid a confounding effect on metabolic and bone parameters relating to medication use (including TRT) or another disease process (including a history of thyrotoxicosis).

Studies that evaluated the prevalence of heterozygous TSHR-Ms (primarily in paediatric cohorts) report a prevalence of ~1:10 amongst TPO negative subjects (Camilot *et al* 2005, Tonacchera *et al* 2004). One paper on adults with non-autoimmune SH and variably raised

TSH (6.6-46mU/L) identified mutations in 4 of 10 subjects, 8 with familial SH (Alberti *et al* 2002).

The SH literature indicated that I might reveal thyroid autoimmunity in between 40% (Surks & Hollowell 2007) and 80% (Allan *et al* 2000) of SH subjects. Thus, assuming that ~60% would be seropositive this would predict identifying ~4 mutation bearing subjects per 100 recruited to the study. It was therefore evident at the planning stage of this project (when anticipated recruitment was ~350), that I was unlikely to have enough mutation subjects to enable meaningful evaluation of their bone parameters. However, I expected to have sufficient numbers to make evaluations according to their TPO antibody status.

In recent years it has been identified that many subjects residing from areas traditionally considered to be iodine sufficient (such as the UK) are not iodine sufficient (Vanderpump *et al* 2011, Bath *et al* 2014). Whilst iodine sufficiency is associated with thyroid autoimmunity, iodine insufficiency is not (Teng *et al* 2011). Consequently I chose to evaluate the iodine status of my participants retrospectively. This was not part of the prespecified study protocol.

The primary objectives of this SH study were as follows;

1. To determine the prevalence of heterozygous TSHR-Ms in an adult cohort residing in the Cardiff area.

2. To evaluate bone parameters (DXA Z-scores at hip and lumbar spine (LS) and BTMs) in TSHR-M subjects versus normal sequence counterparts (albeit within the limitation of low subject numbers).

3. To evaluate bone parameters in TPO antibody positive versus negative subjects.

4. To evaluate bone parameters alongside individual TFT-components, with a particular interest in TSH.

Denaturing High Performance Liquid Chromatography (dHPLC) was the genetic screening tool used to identify subjects bearing TSHR-Ms. Participants were screened across all 10 TSHR exons and anomalous dHPLC waveforms were followed up with direct DNA sequencing. This

identified TSHR sequence variants including TSHR polymorphisms and TSHR-Ms. Given the academic interest in the TSHR D727E polymorphism (in particular) and its suggested influence on TFTs (Gabriel *et al* 1999) and BMD (Van der Deure *et al* 2008, Liu *et al* 2012); I chose to evaluate bone parameters in D727E subjects in a similar way to the mutation subjects.

Study participants had their FOXE1 polyalanine tract length (PTL) polymorphism status determined. The FOXE1 gene is involved in thyroid gland (TG) morphogenesis and mutations in this gene lead to Bamforth-Lazarus syndrome (thyroid agenesis, and cleft lip amongst other syndromic features (Bamforth *et al* 1989). Although FOXE1 PTL polymorphisms have not been evaluated in a dedicated SH cohort previously, FOXE1 polymorphisms are associated with alterations in TSH values (Gudmundsson *et al* 2009, Taylor *et al* 2015). I therefore wished to see if FOXE1 PTL polymorphism TFT-component associations would be revealed in this cohort. This particular study objective was added in to the study while recruitment was underway and was not part of the study protocol.

A range of metabolic measurements were recorded on study participants at the time of their study attendance. This included TFTs, fasting glucose, lipids, blood pressure (BP) and morphometric measurements. Serum BTMs were evaluated on every participant and a DXA bone scan arranged.

These data enable anthropometric, bone (BMD-DXA scores and BTMs) and metabolic parameters to be evaluated according to subject TFT-components, TSHR-M status, TPO antibody status and FOXE1 PTL polymorphism status.

The secondary objectives of the study were as follows:

a) To describe the observational and descriptive characteristics of the cohort (including TPO autoantibody status, TFTs and change in TFTs relative to the initial bloods as well as determine the iodine sufficiency status).

b) Characterise the cohort according to:
    - TSHR-M or polymorphism status
    - FOXE1 PTL polymorphism status

c) Evaluate body composition components (BMD, adiposity) and metabolic parameters according to:
    - TPO autoantibody status
    - TSHR-M or polymorphism status
    - FOXE1 PTL polymorphism status
    - TFT-components
    -

Two hundred and eight adults with primary, treatment naïve SH have been recruited and evaluated in this study. The *primary objectives* of the study were met and reveal the following observations:-

1. **The prevalence of heterozygous TSHR-Ms was ~1:10 amongst TPO negative subjects, and 1:18 within the cohort.**

I identified TSHR-Ms in 12 study participants. The prevalent mutation in this cohort was the W546X, identified in 7 subjects. Previous research from our unit revealed this mutation in 1:180 healthy blood donors (Jordan *et al* 2003) revealing a prevalence 6 times higher in this cohort.

I identified 3 known mutations (W546X, R531W and P162A) and 2 novel mutations. The novel mutations are the Y195C and W488X (in two participants).

The association between TPO seronegativity and TSHR-M status was supported in this study. Eleven of the twelve subjects were TPO antibody negative and one had low measureable antibodies (W488X; TPO antibody titre 60 [range <6 to >1000IU/ml]).

T3 values were lower (relative to TSH and T4) in the TSHR-M group relative to other subjects. A degree of TSHR resistance imposed by the mutation is expected (Tenenbaum-Rakover 2012). Potential explanations for this observation are a negative impact on thyroidal T3 synthesis and/ or reduced peripheral D2 deiodinase activity reducing T4 to T3 conversion. Upregulated DI02 in response to TSH activation is described in skeletal muscle (Hosoi *et al* 1999), rat brown fat (BAT) (Murakami *et al* 2001a) and thyroid tissue (Murakami *et al* 2001b).

2. **No association between TSHR-M status and bone parameters were revealed. However, the study was insufficiently powered to be confident in this observation.**

Although I do not reveal associations between TSHR-M status and bone parameters in this study this does not rule out an effect (albeit if present it is likely to be small and not of clinical relevance). Indeed there are a few considerations worthy of comment.

Firstly given that I observe a negative association between BMD and T3 (across the cohort) and TSH-M subjects appear to have inherently lower T3 (relative to TSH); a positive BMD correlation might have been expected. However, if this is being offset by a direct negative BMD effect from TSHR-M status, then this would support Abe *et al*'s (2003) conclusions associating lower BMD values with TSHR insufficiency.

An alternative perspective is that the negative association between TSH and BMD across the cohort reflects an *indirect* TSH - BMD effect that is independent of TSHR-M status (potentially explaining the absence of an association between TSHR-M status and BMD DXA scores).

3. **A negative association between TPO antibody seropositivity and BMD at the lumbar spine (LS) was revealed.**

This association between BMD and TPO antibody positivity is interesting as it implies that the aetiology of SH is important in regard to its clinical implications. This could be the consequence of inflammatory mediators stemming from the TG (the site of autoimmune injury in ATD) enhancing bone resorption distally in the spine. This mechanism is proposed to explain enhanced bone loss in inflammatory conditions including the spondylarthritides (Akgöl *et al* 2014) and Crohn's disease (Klaus *et al* 2002). Given that the trabecular to cortical bone ratio is higher in vertebrae than hip, and trabecular bone has a higher basal metabolic rate (Clarke 2008) and surface area to mass ratio (Watts 1999). This offers a potential mechanism *contributing* to the negative relationship observed between DXA Z-score and TPO antibody positivity at the LS specifically.

4. **Independent negative associations between TSH and DXA Z-score at the LS, and between T3 and DXA Z-score at the hip and LS were observed.**

A negative relationship between T3 and BMD is well recognised however this is in the context of hyperthyroidism (Lee & Ananthakrishnan 2011) or Shyper, particularly in postmenopausal women (Faber *et al* 1998, Mudde *et al* 1994, Bahn *et al* 2011), but not in SH. What is equally interesting is a positive T3 - TSH relationship revealed across this cohort which is contrary to traditional thinking.

My data suggests that a rise in T3 (which could be accompanied by increased sensitivity to T3) may be contributing to adverse bone effects in SH. This may relate to altered deiodinase enzyme activities (in the thyroid or peripheries), that might be TSH mediated (Hosoi *et al* 1999, Murakami *et al* 2001a and 2001b). The negative BMD - T3 association observed across the cohort suggests that changes to FTHs, even within *normal* population reference ranges may mediate functional effects.

The inverse association between TSH and BMD at LS supports the notion that TSH has adverse effects on BMD in the context of SH (Lee *et al* 2006, Lee *et al* 2010, Polovina *et al* 2013, Liang *et al* 2014). The fact that that my data reveals adverse independent TSH associations at LS but not hip could relate to the differing proportions of trabecular to cortical bone at these sites. Though perhaps, more plausibly there may be enhanced TSHR expression +/- sensitivity +/- potency of effect in the vertebrae versus the hip; potentially explaining this observation.

It is interesting that the BMD associations revealed across the cohort did not correlate with BTM associations. Possible explanations for this were discussed in section 4.9.4.

A negative association between male sex and BMD at hip and LS was observed (Appendix 6, Model 3). Given that testosterone is expected to have a protective effect on BMD (Baran *et al* 1978) and is not expected to potentiate TSH or T3 mediated effects, the explanation for this is obscure. However testosterone levels were not assessed in this study and therefore a confounding effect from altered testosterone levels cannot be ruled out. However it is interesting that Lee *et al* (2010) reported incident hip fracture rates to be elevated in older

(≥65 years) US men with SH (Hazard ratio 2.3 (95% CI 1.25-4.27) but not women. Nonetheless, this observation was unexpected and requires verification and investigation in future studies.

The *secondary* objectives of the study were met and revealed the following observations:

a) Observational data on the cohort

Three quarters of the cohort were female, age distribution showed a negative skew, and 50% were TPO autoantibody positive. These observations were consistent with expectations in SH (Tunbridge *et al* 1977, Fatourechi 2009). The iodine status of the cohort was described as *mildly deficient* (World Health Organisation 2007) supporting recent data identifying iodine insufficiency in regions traditionally considered iodine sufficient (Vanderpump *et al* 2011). My assessment of individual's iodine status (see figure 3.6 and content of pg 78) suggest that iodine excess or deficiency *may* have caused SH in those cases. However, Iodine status did not correlate with TSH levels or TPO antibody status in this cohort.

The TSH fell on retesting in three quarters of the cohort, and back into the reference range (<5mU/L) in just under half. This phenomenon is described for newly identified SH (Diez & Iglesias 2009). However, TSH remained above 3mU/L in 90% and many experts consider a TSH <2.5mU/L to be a true normal (Wartofsky & Dickey 2005). T4 fluctuations on re-testing were much more modest. An expected negative association between T4 and TSH was revealed (Hoermann *et al* 2010), along with the unexpected T3 - TSH relationship (discussed).

Progression of SH to a TSH ≥10mU/L (standard criteria for TRT) occurred in 10% of TPO positive subjects versus 2% of TPO negative subjects. Progression of SH was found to correlate with TPO antibody titre (Figure 3.13). Both of these associations are recognised (Vanderpump *et al* 2005, Zhang Y et al 2010 consecutively).

b) The TSHR polymorphism D727E was identified in 4% of the cohort and an association with TPO seronegativity was revealed.

The FOXE1 14/14 polyalanine tract length (PTL) polymorphism was identified as the most prevalent genotype, whilst the 14/16 was revealed in approximately a third. A positive association between the 14/14 polymorphism and T3 was revealed.

The prevalence of the D727E polymorphism is less than that reported in control cohorts (10-16% prevalence described; Matakidou *et al* 2004, Mühlberg *et al* 2000 respectively). This could possibly reflect its association with lower TSH values (Hansen *et al* 2007), variably described. The association I observe with seronegatively was unexpected and could indicate a linked gene effect. Interestingly an association with FOXE1 PTL$^{14/16}$ status was revealed. However, low subject numbers (n=9) limits my confidence in any of these observations.

The association between FOXE1 PTL status and TFTs indicates that this polymorphism may have functional relevance (presumably exerted in the thyroid gland where it influences the transcription of thyroglobulin and TPO (Francis-Lang *et al* 1992)). Indeed reduced transcriptional activity in the 14/16 relative to the 14/14 genotype is described (Martyn Bullock 2007, Bullock *et al* 2012), and may be relevant to this observation.

c) **TPO antibody positivity associated negatively with BMD at LS. No independent associations with adiposity or any of the cardiometabolic parameters evaluated in the study were revealed.**

   TSHR-M status did not correlate with alterations in body composition components or cardiometabolic parameters (although low subject numbers are a limitation).

   TSHR D727E polymorphism status associated negatively with BMD at LS (on selective matching analysis). Although low subject numbers are a limitation.

   FOXE1 PTL polymorphism status did not show BMD or adiposity associations. However, an unexpected negative association between the 14/14 polymorphism and blood pressure (BP) was revealed.

   TSH did not show independent associations with adiposity or any of the cardiometabolic risk parameters assessed in this study and neither did T4. Positive associations between T3 and BP, and T3 and HOMA-IR were revealed.

Autoimmunity is associated with inflammation which in turn is associated with endothelial dysfunction (a factor in the pathogenesis of atherosclerosis (Steyers & Miller 2014). However my small study did not reveal independent associations between TPO antibody positivity and any of the cardiometabolic-risk parameters assessed.

TSHR-Ms have *not* been associated with altered adiposity or cardiometabolic-risk parameters previously. Given that TSHR-Ms are associated with a degree of functional TSHR resistance, presumably overcome by an appropriate rise in TSH; metabolic effects relating to TSHR-M status may not have been expected.

The negative association between the D727E polymorphism and LS BMD supports the observations of liu *et al* (2012) who reported a higher prevalence of this polymorphism amongst osteoporotic Chinese men versus their non-osteoporotic controls. However, other studies are contradictory (Van der Deure *et al* 2008). Clearly this observation requires verification with much larger subject numbers.

The negative association between the FOXE1 $PTL^{14/14}$ polymorphism and BP is interesting and independent of its T3 association. I hypothesis that as this group have higher T3s relative to 'other' genotypes they may be better adapted in the vascular effects of T3, as reflected in this BP association. This effect could relate to FOXE1 expression in the nervous system +/- liver (based on mRNA extraction from these sources (Genecards human gene database; FOXE1 (www.genecards.org)) thereby affecting vascular tone. However, this observation was unexpected and warrants further investigation.

TSH did not show independent associations with adiposity or any of the metabolic parameters assessed in this study. Given that the majority of subjects in my cohort had TSH levels <10mU/L at study participation (95%); these observations potentially support the notion that with a TSH <10mU/L there is unlikely to be a clinically significant effect on metabolic parameters for the majority of subjects (Parle *et al* 1992, Geul *et al* 1993, Villar *et al* 2007, Garin *et al* 2014, Ye *et al* 2014, Walsh *et al* 2006). However, the cross sectional nature of this study is not designed to adequately address this question.

Interestingly a positive association between T3 and; HOMA-IR and BP was revealed. These associations are recognised in hyperthyroidism (insulin resistance; Maratou *et al* 2009, hypertension; Danzi & Klein 2003), and have even been reported in euthyroidism (Bakker *et al* 1999) but are not described in SH. This again suggests that FTH variance within the normal reference range may be important.

These observations might suggest that adverse cardiometabolic associations that are often reported in SH (Serter *et al* 2004, Iqbal *et al* 2006, Ochs *et al* 2008, Teixeira *et al* 2008, Rodondi *et al* 2010) may be the consequence of a relative rise in T3 (+/-altered T3 sensitivity) rather than relate to altered TSH or T4 levels.

6.1 Summary and the future direction of the study

This SH project is the largest of its kind to evaluate TSHR-M status in an adult cohort, and to evaluate this alongside full thyroid function parameters, TPO antibody status and a range of metabolic and bone related parameters. To my knowledge this is the first study to determine FOXE1 PTL polymorphism status in a dedicated SH cohort. This study has revealed a number of interesting observations.

The key observations from this study are as follows:

Triiodothyronine levels are mildly and positively associated with TSH. This suggests that patients with mild SH (TSH <10mU/L) are not in a state of mild hypothyroidism but are appropriately compensated, or may even be over compensated from a FTH perspective. Interestingly adverse T3 associations were revealed against; BMD, HOMA-IR and BP. This suggests that adverse metabolic associations variably reported in SH may relate to a relative rise in free-T3.

Negative BMD associations were revealed against TSH, TPO antibody positivity and male sex. These observations suggest that SH may have negative effects on BMD that are more relevant to subjects with autoimmune SH, additional risk factors for osteoporosis, and men. Should these observations be verified in future studies then active evaluation of bone health, in these groups will be indicated.

I suggest that future prospective studies of adequate duration and power are planned to evaluate bone parameters in subjects with and without SH, and with and without thyroid autoimmunity. In addition studies to determine whether the natural history of SH (in regard to bone health) is altered in the event of its optimum correction (with TRT) are indicated. However, the practicality of performing these studies, on the scale required may be difficult.

TSHR-Ms were found to affect 1:10 TPO negative subjects and were found to reside as expected, and overwhelmingly in the TPO negative cohort. A negative association between TSHR-M status and T3 was revealed although this did not translate into obvious metabolic or BMD associations. Two novel TSHR-Ms were identified in this study (W488X (in 2 subjects) and the Y195C).

The FOXE1 PTL polymorphism data revealed a positive association between the prevalent 14/14 PTL polymorphism and T3, and an inverse association between this genotype and blood pressure. These observations require verification in future cohort and population studies.

6.2 Limitations of the study

- The number of subjects recruited to the study (13% of those identified by the department of biochemistry) fell short of those anticipated (208 recruited versus 350 anticipated) reducing the power of the study to determine its objectives.

- Recruitment was *un-randomised* creating opportunity for *selection bias*. Therefore the cohort may be poorly representative of the SH population (i.e. more or less diseased, or biasing observations towards or away from the null hypothesis).

  The major sources of bias were;-

  - Poor and sporadic GP participation across the region.
  - *Subject choice* to participate

- The fact that nearly half the subjects recruited to the study had TFTs at study attendance within the normal TSH range could be considered a limitation. However

this limitation applies to all cross sectional SH studies where correlations are made against TFT values taken at a point in time, which indicate SH.

- The heterogeneity of the cohort (i.e. age, comorbidities, lifestyle factors and morphometric characteristics) reduces the power of the study to determine differences that relate specifically to the parameters of interest.

- As the study design involved a one stop study attendance I am limited in assessing trends across the cohort. Evaluating intra-individual variation with time, or comparing observations against a well matched, euthyroid control group would have been a more powerful means of exploring these objectives.

- The statistical observations made on this cohort were assessed against TFTs taken at the time of study attendance. The study, by its nature assumes these values are representative of the subject's TFTs over a considerable length of time. However, a substantial change in TSH values (particularly) was commonly seen (Figure 3.15).

- BTMs are known to display wide inter-subject variability thereby limiting their utility as a single point assessment tool (Burch *et al* 2014). This may explain the absence of observed BTM associations in this cohort.

- BP was assessed at a single appointment session and may be poorly representative of the subject's usual BP.

- It is unfortunate that the TFT and TPO assay changed ((from *Siemens Centaur* to *Abbott i2000R*) after the 139th subject had been recruited to the study. As the T4, T3 and TPO antibody results were not convertible between assays, all former samples were re-evaluation on the Siemens Centaur assay (from stored and frozen samples). Instead, subject numbers >139 were evaluated fresh on the day of study attendance. These differing conditions surrounding the T4, T3 and TPO antibody assessments may have introduced selective error in to the results obtained.

- The TSH values on the Siemens Centeur and Abbott Architect *i2000R* did appear to be readily convertible between assays (see Appendix 8). However the fact that only 40 samples were evaluated to generate the data is a limitation. In addition the conversion recommendation between assays is an imprecise estimate. As this conversion was only required for subject numbers >139, selective error is introduced in to the TSH results obtained.

- The Insulin values (for HOMA-IR evaluation), like the TFTs (in subject numbers <140) were assessed from stored and frozen serum that had been in storage for variable durations (months to years). It is likely that degradation of serum may have occurred (possibly aggravated by the freeze-thaw process) potentially affecting the reliability of these results.

- The lifestyle information recorded at study attendance (I.e. activity levels, alcohol intake per week, smoking history) was provided by the subject and may not be exact. However, these parameters were categorised into broad groups for evaluation (Activity; sedentary versus non-sedentary, Alcohol; abstinent versus non-abstinent, Smoking; more than 10 pack year history versus not) reducing subjective error.

- There was a variable delay in the DXA scan being performed post study attendance (same day to up to several months later). However, as BMD is expected to change slowly with time (over years) I would not anticipate a detrimental effect relating to this arrangement. DXA values were reported against White, Oriental or Afrocaribbean datasets (more ethnic-specific reference data was not available).

- DXA Z-scores were used in this study as a surrogate indicator of bone health and fracture risk. However, BMD is one of many factors affecting fracture risk (i.e. age, BMI, balance, strength, co-morbidities, medication and alcohol use etc (Hippisley-Cox & Coupland 2009). The limitation of DXA as a fracture risk tool is exemplified by the fact that ~50% of women with vertebral fractures have lumbar BMD in the normal range (Kanis *et al* 2006).

- The study was greatly underpowered to determine BMD differences relating to TSHR-M (n=12) or TSHR D727E polymorphism status (n=7). This related to their low prevalence, wide DXA Z-score standard deviations, and suboptimal cohort recruitment. Thus, to demonstrate a Z-score difference of 0.4 as statistically significant on MRA; a cohort of ~580 subjects are required assuming a significance at 5% and power of 80% (predicts ~30 with the DNA sequence of interest).

- As a consequence of low subject numbers I chose to evaluate the TSHR-M and TSHR D727E groups using a case-selected control method. However, the matching process was compromised through having relatively few suitable matches available and was un-randomised and vulnerable to selection bias.

- The novel TSHR-Ms that were identified (in 3 of 12 subjects) have not been functionally assessed. The nonsense mutation (W488X x2 subjects) is expected to affect function whereas the missense mutation (Y195C) may not. Thus, although I consider these subjects as a unified group for evaluation purposes, functional studies on the novel mutations (particularly Y195C) are indicated.

- The original study protocol differed from the actual study performed in several respects;-

a) The original intention was for the TPO +ve subjects to be compared against TSHR mutation subjects, as the causes of their SH differed. However, the actual study compared the TPO +ve subjects with TPO -ve subjects, and TSHR mutation subjects with normal TSHR sequence subjects (as these were considered to be more suitable comparative groups). However, this fundamental change to the study design (thereby limiting the application of the prespecified study protocol and power calculations) may compromise the study in its ability to robustly evaluate its principle objectives.

b) As there was no pre-specified plan to identify (or evaluate) subjects bearing TSHR or FOXE1 PTL polymorphisms; the observations on these sub-groups should be considered in *hypothesis generation* only.

c) These changes to the study resulted in a greater number of statistical tests being performed, thereby increasing the likelihood of type 1 errors occurring.

Despite these limitations I feel that this has been a valuable and informative project. The observations I have made will provide a platform for future research; potentially enabling a more precise understanding of the physiology, pathophysiology and heterogeneity of SH to be made.

I hope that this work might ultimately contribute towards improvements in the clinical care of subjects with subclinical hypothyroidism.

APPENDIX 1

GIG CYMRU NHS WALES
Bwrdd Iechyd Prifysgol Caerdydd a'r Fro
Cardiff and Vale University Health Board

University Hospital of Wales
Ysbyty Athrofaol Cymru

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ORIGINAL STUDY PROTOCOL

Title: Effects of a single functional TSH receptor on bone metabolism and body composition

Cardiff University reference: 08/CMC/4402

Contents:

List of Abbreviations

BMD	Bone mineral density
BMI	Body Mass Index
DXA	Dual energy x-ray absorptiometry
DHPLC	Denaturing high performance liquid chromatography
HDL	High density lipoprotein
LDL	Low density lipoprotein
SH	Subclinical hypothyroidism
TPO	Thyroid peroxidase
TRAB	TSH receptor antibodies
TSHR	Thyroid Stimulating Hormone receptor
UHW	University Hospital of Wales

1. Title: Effects of a single functional TSH receptor on bone metabolism and body composition

2. Introduction

Subclinical hypothyroidism (SH), defined as an elevated thyroid stimulating hormone (TSH) in the presence of normal circulating levels of free thyroxine (T4) and free tri-iodothyronine (T3), is a common condition affecting 3-6% of the UK population (1, 2). Most cases have an autoimmune basis (chronic lymphocytic thyroiditis) with 50-80% of patients possessing anti-thyroid peroxidase antibodies (3). In these individuals, SH is frequently a prelude to overt hypothyroidism with conversion rates of up to 5% annually, especially in individuals with higher levels of TSH (>10mU/l) (4). However, treatment of SH remains controversial, especially for individuals with lower degrees of TSH elevation. Although the data are largely consistent for modest beneficial effects of thyroxine on the lipid profile, cardiac and vascular function in SH, symptomatic benefit is less well established (3) and the effects of SH on bone health have not been widely studied. Current recommendations therefore support thyroxine replacement only in subjects with higher levels of TSH (>10 mU/l) (5).

Much recent interest has focused on the effects of TSH on the skeleton and body composition. Thyroid hormone excess has long been known to accelerate bone remodelling, leading to negative calcium balance and bone loss (6) which, at least in overt thyrotoxicosis, may be associated with increased risk of osteoporosis and fractures (7). This has traditionally been viewed as a consequence of thyroid hormone action alone, in line with data from some animal models which suggest that TSH has little or no bearing on bone turnover (8). However, a recent publication has challenged this assertion, indicating that TSH is itself a negative regulator of skeletal remodelling (9). The authors produced mice null for the TSH receptor (TSHR) and found that even the heterozygotes displayed profound osteoporosis with focal osteosclerosis. Subsequent *in vitro* experiments demonstrated inhibition of osteoclast production and survival, combined with inhibition of osteoblast differentiation, by TSH. The absence of these mechanisms in the TSHR $^{-/-}$, and their reduction in TSHR $^{+/-}$ mice, explains the phenotype.

It is not known whether TSH, via its receptor, has a similar action on bone metabolism in humans, although recent data from a large population-based study in the Netherlands supports an important independent action of TSH on bone mineral density (BMD) (10). In this study, BMD at the femoral neck and cortical thickness increased with serum TSH, a response only partly driven by increased body mass index (BMI). Furthermore, carriers of a Glu^{727} polymorphism in the TSHR, which is associated with higher receptor activity as less TSH is

required to maintain normal T4 levels (11), showed a 2.3% increase in femoral neck BMD (10). Our own observations in the clinic (unpublished data) of an osteoblastoma on the forearm of a child homozygous for the TSHR nonsense mutation W546X (equivalent to $TSHR^{-/-}$ mice), is intriguing and adds further weight to the notion that loss of TSHR function may have important consequences on bone function in humans.

We have previously shown that heterozygosity for the W546X variant is common in the local general Caucasian population, occurring at an estimated prevalence of 0.5% (12). This mutation causes TSH resistance with a biochemical phenotype usually typical of SH, namely normal T3 and T4 with elevated TSH (12-14). Screening for germline mutations in the TSHR in other populations with SH supports a comparatively high prevalence in the antibody negative subgroup, variably estimated at 12-40% (15, 16). These reports indicate that possession of a single functional TSHR can cause SH and that an allele producing this genotype is common in the UK. This suggests that many SH patients are the equivalent of the $TSHR^{+/-}$ mice and are at risk for the development of osteoporosis. If confirmed, this finding would not only improve our understanding of the molecular basis for osteoporosis, a disease which accounts for over 200,000 fractures and an annual health service cost of £1 billion in the UK, but would also have immediate relevance to the management of patients with SH and other thyroid disorders. This might include avoidance of thyroxine replacement in antibody-negative patients with SH found to have a single functioning TSHR, because attempted TSH lowering with T4/T3 could further worsen an already lowered BMD.

In light of these observations, we postulate that loss-of-function of a single allele of the TSHR is a common cause of SH in the UK but may have important phenotypic differences from autoimmune SH in relation to bone physiology. We aim to test this hypothesis in our local population.

3. Aims

1. To determine the proportion of SH due to TSHR heterozygosity by genotyping.

2. To assess bone metabolism in SH patients expressing one loss-of-function TSHR allele and compare it with SH patients who develop autoimmune thyroiditis, using biochemical markers of bone turnover and measuring bone mineral density (BMD).

4. Investigational Plan

Study overview

Patients with *de novo* SH and no known history of previous thyroid disease or treatment will initially be identified via the Department of Medical Biochemistry and invited, via their GP or referring physician, to participate in the study. Subjects who indicate a willingness to participate and who meet the eligibility criteria will be asked to attend the Clinical Research Facility (CRF) at the University Hospital of Wales (UHW) for further review. After obtaining consent, pre-menopausal women will be tested for pregnancy (excluded if positive) and all patients will undergo simple anthropometric measurements (height, weight, waist circumference and standard bioimpedance for assessment of body composition), blood and urine testing (stored for later measurement of markers of bone turnover). Blood will also be drawn for repeat measurement of thyroid function (free T4, free T3, TSH) and antibody status (anti-thyroid peroxidase and thyroid blocking antibodies, TBAB) according to routine clinical practice. Subjects will then undergo a standard DEXA scan for measurement of bone mineral density (BMD) at the lumbar spine and left hip. The cohort will subsequently be split into two groups: those with positive anti-thyroid antibodies (autoimmune thyroiditis; expected to be approximately 2/3 of the total) will form the control group while the remaining patient samples will undergo TSH receptor (TSHR) genotyping to search for loss-of-function mutations (expected to occur at a frequency of ~40% in the antibody negative subjects). Any novel mutations will be characterised using established *in vitro* protocols; allele frequencies will also be determined in the antibody positive group. DEXA scores, bone biochemical markers and body composition measurements will finally be compared between subjects harbouring one mutant TSHR allele (approximately 50 over a two year period) and TPO antibody positive controls (~250), with appropriate adjustment for age, gender and BMI.

Study population/inclusion and exclusion criteria

Patients will be identified via the Biochemistry department at the UHW. A recent audit reported that approximately 250 new patients with SH are detected at UHW per annum, once those with known thyroid disease or receiving thyroid medication are excluded. Assuming two years recruitment and a 70% participation rate we would expect to generate a cohort of ~350 individuals of whom 2/3 will be TPO antibody positive (control group). The remaining 120 individuals will undergo TSHR genotyping, of whom we would expect ~40% (50 subjects; affected group) to harbour a single functioning TSHR allele (16).

Inclusion criteria

- Male or female patients aged between 18 and 70 years (on screening bloods)
- Patients with SH as demonstrated by a normal free T4 and elevated TSH (of any degree)
- Patients for whom written informed consent to participate in the study has been obtained prior to any study related activity

Exclusion criteria

- Subjects aged <18 or >70 years (on screening bloods)
- Pregnancy and breastfeeding
- Pre-existing thyroid disease treated with surgery, radioiodine therapy or drugs (carbimazole, propylthiouracil, thyroxine or tri-iodothyronine)
- Metabolic bone disease: hypo/hyperparathyroidism, vitamin D deficiency, Paget's disease
- Osteoporosis
- Endocrine disease affecting BMD: Hyperprolactinaemia, Cushing's syndrome, Acromegaly, hypopituitarism (of any degree), hypogonadism
- Coeliac disease
- Malabsorption
- Anorexia nervosa
- Chronic renal failure
- Chronic liver disease
- Cystic Fibrosis
- Inflammatory bowel disease
- Currently taking or recent (within 6 months of study entry) exposure to: glucocorticoids, androgens, antiandrogens, glitazones, cyclosporin, chemotherapy, anticonvulsants, vitamin D or depot contraceptive preparations (e.g. depo-Provera). Women taking oral contraceptives or oestrogen replacement therapy will be eligible to participate but these variables will be adjusted for in the final analyses
- Any previous treatment with bisphosphonates, raloxifene, strontium or recombinant PTH

Investigational measurements

Subjects will attend the CRF at 0800 hours in a fasted state. Informed consent will initially be obtained and all pre-menopausal women will be tested for pregnancy; any found to be pregnant will be excluded from further participation. Eligibility criteria will be reviewed and patients will be asked about previous medical history (including fractures), medication, smoking status, alcohol intake, exercise history and family history of thyroid disease or osteoporosis. Three blood pressure measurements will be obtained and averaged using a validated OMRON sphygmomanometer. Body height (without shoes) will be measured to the nearest 0.5 cm using a stadiometer and body weight (in light clothing without shoes) to the nearest 0.5 kg on a balance beam scale. Waist circumference will be measured at minimal respiration and reported to the nearest 0.5 cm by positioning a flexible anthropometric tape parallel to the floor and immediately above the iliac crest. Hip circumference will be measured at the maximum circumference over the buttocks and recorded to the nearest 0.5 cm. Body mass index (BMI) will be calculated as weight (kg) divided by height (m) squared. Waist:hip ratio (WHR) will be calculated as waist circumference divided by hip circumference. Standard bioimpedance will be used to determine lean body mass, fat mass and fat percentage (Tanita bioimpedance meter).

Approximately 20 ml of blood will be drawn from the antecubital vein for the measurement of thyroid function (free T4, free T3, TSH), thyroid antibodies (anti-thyroid peroxidase, thyroid blocking antibodies (TBAB)), bone profile (calcium, phosphate, alkaline phosphatase), total cholesterol, HDL, LDL, triglycerides, glucose, insulin and TSHR genotyping. Serum will also be saved (frozen at -30°C) for later batched analysis of osteocalcin, bone-specific alkaline phosphatase, procollagen type 1 aminoterminal propeptide, C-cross-linking terminal telopeptide of type 1 collagen and tartrate specific acid phosphatase (TRAP). A urine sample will be obtained for measurement of free deoxypyridinoline and N-telopeptide of collagen cross-links.

For the TSHR genotyping the patients will be screened initially to detect markers of autoimmune or atrophic thyroiditis including antibodies to thyroid peroxidase and thyroid blocking antibodies (TBAB) both using in-house assays. To our knowledge, we are the only academic centre in the UK able to assay for the full spectrum of thyroid autoantibodies, including their biological function.

We would predict that approximately two-thirds of the cohort would be positive for thyroid antibodies and will genotype the TSHR in the remaining ~120 patients. Initially, to estimate the allele frequency of the W546X mutation in the thyroid antibody negative SH patients, we

will genotype individuals, using single nucleotide primer extension and an ABI 3100, as previously described (12-14). Subsequently, for those patients who do not express W546X, we will use Denaturing High Performance Liquid Chromatography (dHPLC). We will screen the entire coding region and about 1000 bp of 5' flanking sequence, which will include the promoter. Since DHPLC uses fragments of 400-500 bp, 13 separate fragments will suffice to cover the promoter, all 10 exons and intron/exon boundaries of the gene.

We have extensive experience in genotyping the entire TSHR in individuals by conventional methods and of large-scale genotyping using DHPLC (12-14).

If we find any novel TSHR mutations their function, in terms of TSH binding, signal transduction and surface expression will be compared with the WT TSHR. We have previous experience in producing the required expression vectors, their transfection (both stable and transient) and functional comparison with WT receptor. In addition, we will genotype the 240 antibody positive SH patients for any TSHR mutations, to assess their allele frequencies.

BMD will be evaluated by dual energy x-ray absorptiometry (DXA) using a Hologic Discovery A absorptiometer. The following regions will be examined: (a) lumbar spine (L1, L2, L3 and L4 separately and total), (b) hip (femoral neck, greater trochanter and inter-trochanteric region individually and total). Given the phenotype of the TSHR $^{+/-}$ and $^{-/-}$ mice, it is possible that changes in bone density will not be uniform. Such heterogeneity can be detected by detailed examination of the localised sites. Quality assurance will be assessed using a Hologic spine phantom, measured once prior to all DXA measurements. In addition to absolute BMD (expressed in g/cm^2) DXA results will also be interpreted according to BMD expressed as a T score, giving the standard deviation score relative to mean peak bone mass in young adults, and the Z score relative to age- and gender-matched controls. The World Health Organisation (WHO) definitions will be used to classify subjects as normal (T-score value for BMD > -1), low bone mass/osteopenia (T-score value for BMD <-1 but >-2.5) or osteoporosis (T-score value for BMD <-2.5).

The total radiation dose is estimated as 11μSv (or the equivalent of about 2 days of background radiation exposure in the UK).

Endpoints

Primary endpoints

The primary endpoints of the study will be differences in BMD (total or local) at the lumbar spine and left hip in the affected (mutation positive) group compared with controls (autoimmune SH).

Secondary endpoints

Secondary endpoints will include percentages of subjects in each group meeting WHO definitions of normal, low bone mass and osteoporosis; serum and urine markers of bone turnover, lipids, insulin resistance (measured by HOMA-IR) and body composition.

5. Data management

Sample size calculations and statistical analyses

Sample size calculations have been performed in conjunction with Professor Robert Newcombe (Department of Primary Care and Public Health, Centre for Health Sciences Research, Cardiff University). A comparison of 50 ‘affected’ patients with 250 TPO antibody positive ‘controls’ detects a shift of 0.44 SDs with power 80% at the conventional 5% alpha level. Comparisons of continuous variables between groups will be performed by unpaired t-test or Mann-Whitney U test for normally or non-normally distributed variables respectively. Chi square tests will be used to compare distribution of categorical variables. Since the test and control groups will both be derived from individuals with SH, who by definition have 'normal' circulating T4 and T3, we do not anticipate any confounding effects due to variation in thyroid hormone levels, but analyses will be adjusted if necessary for these and other potential confounders such as age, gender and BMI.

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7. Project management

Dr Anna de Lloyd (Clinical Research Fellow) will be responsible for the day-to-day conduct of the study. She will co-ordinate recruitment, obtain consent, undertake the blood and urine sampling, perform the bioimpedance analysis and perform the TSHR genotyping in conjunction with colleagues in the Centre for Endocrine and Diabetes Sciences (supervised by Dr Marian Ludgate, Reader) and Department of Medical Biochemistry (Dr Carol Evans, Dr Hilary Durrant). She will also be responsible for data entry and storage and will at a later stage conduct statistical analysis on the data obtained, in conjunction with Dr Rees and Professor Robert Newcombe from the Department of Primary Care and Public Health, Centre for Health Sciences Research, Cardiff University.

Miss Sarah Darlington, Mrs Rebecca Pettit and Dr Will Evans (Department of Medical Physics, University Hospital of Wales) will perform the DEXA measurements. Dr Aled Rees and Dr Marian Ludgate, the Principal Investigators, will act directly as Dr de Lloyd's supervisors. A formal meeting will take place on a weekly basis to discuss all aspects of the study and to plan the work for the forthcoming week. Dr Rees and Dr Ludgate will ensure that Dr de Lloyd is trained in all the necessary clinical and laboratory procedures, including attendance at a Good Clinical Practice training day. Informal discussions regarding any difficulties that arise with the project will also take place as required.

A further monthly meeting involving Dr de Lloyd and Dr Rees/Dr Ludgate will occur to discuss overall progress and to focus on manuscript/abstract preparation as the results allow.

Study data will be stored for 15 years after completion of the study. Dr Rees will act as custodian of these data and only Dr Rees, Dr Ludgate and Dr de Lloyd will have access to the data.

8. Administrative Procedures

Changes to the protocol

Any change or addition to this protocol will require a written protocol amendment. Amendments significantly affecting the safety of subjects, the scope of the investigation or the scientific quality of the study will be submitted for additional approval by the Local Research Ethics Committee and the Research and Development departments at Cardiff and Vale NHS Trust and Cardiff University.

Auditing procedures

As part of Good Clinical Practice, Dr Rees will ensure that the study protocol and documentation are closely monitored. The study will be conducted as outlined in the study protocol and in accordance with all applicable government regulation. All study documentation will be available for inspection at any time by appropriate regulatory authorities including internal audits by the Cardiff and Vale NHS Trust and/or Cardiff University Research and Development audit officers.

Publication of results

Any formal presentation or publication of data from this study will be considered as a joint publication by the investigators and authorship will be determined by mutual agreement. The research findings will be disseminated through peer reviewed publications and presentations at regional/national/international meetings. Following scientific peer review and publication, the results of the study will be communicated directly to all participants and, additionally, to local patient support groups where appropriate.

9. Consent

Potential participants will initially be identified by members of the patient's direct healthcare team, specifically Dr Carol Evans and Dr Hilary Durrant, Department of Medical Biochemistry, UHW. Drs Evans and Durrant are responsible for the daily validation of patients' thyroid function test results and will screen all patients identified as having SH for evidence of previous thyroid disease or prior or current thyroid hormone or anti-thyroid drug treatment, where this information is available; these patients will not be considered for this study. The remaining patients, estimated to be approximately 5 per week, will be assumed to have *de novo* disease.

The referring GP or hospital physician responsible for their care will then be contacted by Dr Evans or Durrant via telephone and letter. This initial letter, which will enclose a patient information sheet, will outline the principles of the study and will request that the GP/physician contacts the patient to make them aware of the study and to enquire whether they might be willing to consider participation. Recognising that many practitioners would prefer referral to an Endocrinologist, the letter will also offer rapid review in Dr Rees' clinic as an alternative. The patient information sheet will explain the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail. Each subject will be informed that participation in the study is voluntary and he/she may withdraw from the study at any time and that withdrawal of consent will not affect his/her subsequent medical treatment or relationship with the treating physician. Patients will be given 1 week to review the patient information sheet and will be provided with a contact number for the study team such that they can ask questions about any aspects of the study. Patients who express an interest in participation will subsequently be invited to attend the CRF at the UHW where the consent process will take place. The following individuals will be permitted to take consent:

Dr Anna de Lloyd (Clinical Research Fellow)

Dr Aled Rees (Principal Investigator)

This informed consent will be given by means of a standard written statement, written in non-technical language. The subject should read and consider the statement before signing and dating it, and will be given a copy of the signed document. If the subject cannot read or sign the documents, oral presentation may be made or signature given by the subject's legally appointed representative, if witnessed by a person not involved in the study, mentioning that the patient could not read or sign the documents. No patient can enter the study before his/her informed consent has been obtained.

APPENDIX 2

GIG CYMRU NHS WALES
Bwrdd Iechyd Prifysgol Caerdydd a'r Fro
Cardiff and Vale University Health Board

University Hospital of Wales
Ysbyty Athrofaol Cymru

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☏ *029 20742341* , 🖷 *029 2074 4671*

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Minicom 029 2074 3632

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Bychan
Caerdydd CF14 4DW
Ffôn 029 2074 7747
Minicom 029 2074 3632

INITIAL LETTER OF APPROACH

Effects of a single functional TSH receptor on bone metabolism and body composition

Dear Dr…… Regarding …….....

You recently requested a thyroid function test on the above patient which has been analysed and shows evidence of subclinical hypothyroidism (SH)

Result Free T4……..(pmol/l)

TSH……(mU/l)

We are currently conducting a study examining the prevalence of mutations in the TSH receptor gene as a cause for SH and how this may influence bone mineral density. We wish to recruit subjects with **primary, treatment naive Subclinical hypothyroidism (SH)**. The details of the proposal are outlined below:

SH, defined as an elevated thyroid stimulating hormone (TSH) in the presence of normal circulating levels of free thyroxine (T4) and free tri-iodothyronine (T3), is a common condition affecting 3-6% of the UK population. Most cases have an autoimmune basis (chronic lymphocytic thyroiditis) with 50-80% of patients possessing anti-thyroid peroxidase antibodies. In these individuals, SH is frequently a prelude to overt hypothyroidism with conversion rates of up to 5% annually, especially in individuals with higher levels of TSH (>10mU/l). However, treatment of SH remains controversial, especially for individuals with lower degrees of TSH elevation, with current recommendations supporting thyroxine replacement only in subjects with higher levels of TSH (>10 mU/l).

We, and others, have recently shown that mutations in the TSH receptor (TSHR) gene may be a relatively common cause of SH in patients who are negative for thyroid antibodies, affecting up to 40% of this group. However, this requires confirmation in larger studies. Furthermore, recent data from animal studies and a large study of middle-aged volunteers in

the Netherlands suggest that the TSHR may have an important role in protecting against osteoporosis independently of thyroid hormones.

Patients with SH whose disease is caused by mutations in the TSHR gene will only have one functional TSHR allele, in contrast to patients with autoimmune SH who possess two working copies of this gene. We hypothesise that patients with 'mutation SH' may have lowered bone mineral density and aim to test this by comparing bone metabolism in SH patients whose disease is caused by mutations in the TSHR gene with autoimmune SH patients, using biochemical markers of bone turnover and measuring bone mineral density (BMD).

Subjects who choose to participate in our study will undergo the following investigations:
Anthropometric measurements (height, weight, waist circumference, blood pressure, bioimpedance)

DXA scan

Blood tests (markers of bone turnover, lipids, insulin, glucose, C-reactive protein, thyroid function tests, thyroid autoantibodies and TSH-receptor mutation analysis)

Urinalysis for markers of bone turnover.

As we are not directly involved in your patient's care we would be grateful if you would be willing to approach your patient to discuss this study with them, perhaps when they attend for review of the above test result. We enclose a patient information sheet which contains the details of the measurements proposed and the contact details of the study team should your patient wish to participate. Alternatively, we also recognise that many clinicians would prefer for their patients with SH to be reviewed by an Endocrinologist. If you would prefer this please refer your patient to Dr Aled Rees, Senior Lecturer in Endocrinology (study Principal Investigator), UHW, who will arrange prompt review and treatment if indicated.

We enclose a tear-off slip and stamped addressed envelope.

Participation in this study will not affect your patient's routine care and patients will be free to withdraw from this study at any time. Should you have any queries regarding this study, please do not hesitate to contact ourselves or Dr Aled Rees at the University Hospital of Wales on either of the following numbers:

02920 745002 (Dr Rees) or 02920 748367 (Dr Carol Evans)

Yours sincerely,

Dr Carol Evans Dr Hilary Durrant

Department of Medical Biochemistry

……Cut here

Name and address of patient

……

……

……

Name and address of GP

……

……

……

Please tick appropriate box

- ☐ I have provided my patient with a copy of the patient information sheet
- ☐ The patient is not suitable for the study
- ☐ I give permission for this patient to be approached regarding this study
- ☐ The patient does not wish to participate in the study
- ☐ I want an outpatient appointment with Dr Rees to be arranged

Please return in the enclosed envelope to: Dr Carol Evans, Department of Medical Biochemistry, University Hospital of Wales, Heath Park, Cardiff

APPENDIX 3

GIG CYMRU NHS WALES
Bwrdd Iechyd Prifysgol Caerdydd a'r Fro
Cardiff and Vale University Health Board

University Hospital of Wales
Ysbyty Athrofaol Cymru

Eich cyf/Your Ref:
Ein cyf/Our Ref: DAR/JE
Welsh Health Telephone Network 1872
Direct line/Llinell uniongyrchol
☏ 029 20742341 , 🖷 029 2074 4671

Heath Park
Cardiff CF14 4XW
Phone 029 2074 7747
Minicom 029 2074 3632

Parc Y Mynydd
Bychan
Caerdydd CF14 4DW
Ffôn 029 2074 7747
Minicom 029 2074 3632

Effects of a single functional TSH receptor on bone metabolism and body composition

PATIENT INFORMATION SHEET
(Version 2 December 2008)

PART 1

1. Title of study

TSH receptor and bone

2. Introduction

You are being invited to take part in a clinical research study. Before you decide whether you wish to become involved it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Please do not hesitate to ask us if there is anything that is not clear or if you would like more information. Take time to consider whether or not you would wish to take part.

Thank you for reading this.

3. What is the purpose of this study?

Underactivity of the thyroid gland ('hypothyroidism') is a common condition affecting 2-5% of the UK population. Sometimes this is very mild and identified only on blood testing (a condition called 'subclinical hypothyroidism' or 'SH'). Most patients who develop SH (roughly 2/3) do so because of antibodies destroying the thyroid tissue which can be measured by a simple blood test ('autoimmune SH'). A decision to treat or simply monitor thyroid function tests (TFT) is often difficult but patients with significant symptoms and signs of more severe thyroid underactivity may be treated with thyroid hormone ('thyroxine').

We have recently found that some patients with blood tests showing SH, but whose antibody tests are negative, have this condition because of a change in the code ('mutation') in a thyroid system gene (called the 'TSH receptor' gene).

We wish to test how common these mutations are as a cause of SH and also to determine whether patients with mutations as a cause for SH have any reduction in bone thickness (bone mineral density) compared with patients with autoimmune SH.

4. Why have I been chosen?

You have been chosen for this study as your recent blood tests have shown that you have SH. A total of 350 patients will be studied.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

6. What will happen to me if I take part?

The study will take place at the Clinical Research Facility (CRF) and the Department of Medical Physics at the University Hospital of Wales. You will be asked to attend in the morning having only had water to drink (i.e. no breakfast, tea or coffee). Each visit will last approximately one and a half hours.

If you are a woman of child-bearing age we will firstly require you to have a pregnancy test before carrying out any measurements.

We will then measure your weight, height, blood pressure, waist circumference, hip circumference, fat percentage (on an instrument called a bioimpedance machine) and take a brief clinical history.
We will take blood samples (approximately four tablespoons) to check levels of 'bone' proteins, thyroid function tests, thyroid antibodies, cholesterol, insulin, glucose and a further sample which we will use to test your TSH

receptor gene. We will also measure a urine sample for markers of bone metabolism.

Finally we will measure the density of your bones (bone mineral density; BMD) using a technique called a DEXA scan. This is a simple, rapid and non-invasive technique which is used routinely in clinical practice to measure BMD at the hip and over the spine. This scan does involve exposure to a small amount of radiation but the risk is negligible.

7. Expenses and payments

If you wish we will be able to reimburse any travelling expenses / car parking fees incurred while attending for the study visit.

8. What do I have to do?

It is important that you take your regular medication in the normal way on the day of study attendance (unless we advise otherwise).

There are no lifestyle or dietary restrictions and you can continue your daily activities normally. We request that you report any illnesses to us as they may influence the timing of your test visit.

You should inform us if there is any possibility of you being pregnant and this will be tested for in all women of a child-bearing age. If you are pregnant you will not be able to participate in this study.

For the study visit, we ask that you attend the CRF at 8 o'clock in the morning having fasted from midnight the previous night. You can drink water freely up to this point.

9. What are the possible disadvantages and risks of taking part?

Other than possible discomfort (temporary pain, swelling, bruising and rarely infection) caused by the collection of blood, no other side effects are anticipated from the study procedures, though as outlined above, a DXA scan does involve exposure to a small amount of radiation but the risk is negligible. It is possible that the blood tests or DXA scan could by chance pick up an unsuspected abnormality, in which case you will be given an opportunity to discuss these findings further with Dr Aled Rees.

The genetic blood sampling will test for variation in your TSH receptor gene. These changes occur commonly in the general population and currently have no known effects other than to give a similar thyroid hormone pattern in the blood as SH. The samples will be fully anonymised and the results of these genetic tests will have no implications for you in terms of insurance status.
If you are found to have a 'mutation' in the TSH receptor gene you will be invited to attend a further appointment with Dr Aled Rees to discuss this in greater detail.

10. Are there any implications for members of my family?

Because mutations in the TSH receptor gene can be inherited, if you are discovered to have a mutation it is possible that other members of your family might also be affected, leading to mild underactivity of the thyroid gland or 'SH'. If you are found to have a mutation we will therefore ask for your permission to contact your immediate family members (parents, brothers/sisters, children) to see whether they would also wish to be tested. There are currently no effects known from TSH receptor mutations other than causing underactivity of the thyroid.

11. What are the potential benefits of taking part?

There are unlikely to be any direct benefits for you but the study may provide us with important information in determining whether changes in the TSH receptor gene are a common cause of SH and whether these are associated with a lower BMD and potential risk for development of osteoporosis in the future.

12. What if something goes wrong?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

13. Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

This completes Part 1.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

PART 2

1. What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, we will tell you and discuss whether you should continue in the study. If you decide not to carry on, we will make arrangements for your care to continue. If you decide to continue in the study we may ask you to sign an updated consent form.

2. What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal. A decision to withdraw at any time will not affect the standard of care you receive.

3. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact number 02920 745002).
This study is being indemnified by Cardiff University. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

4. Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. With your permission your GP will be informed of your participation in this study. With your permission we may also look at sections of your medical notes which are relevant to the research study.

5. What will happen to any samples I give?

The blood samples will be collected and stored securely for later analysis in the Centre for Endocrine and Diabetes Sciences at the UHW. Only immediate members of Dr Rees' research team will have access to these samples. All identifiable information will be removed from the samples which will be destroyed by incineration once the tests are complete.

6. What will happen to the results of the research study?

The results of the research study will be prepared for publication in appropriate medical journals together with presentation at medical conferences. Patients participating in the study will be able to obtain a copy of the results after they have been published in the relevant journal(s). Patients will not be identified in any report/publication.

7. Who is organising and funding the research?

The study is being organised by Dr Aled Rees (the Principal Investigator) and Dr Anna de Lloyd (Research Registrar) from the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales. Funding for the study

is provided from funds within the Centre for Endocrine and Diabetes Sciences at the UHW. The doctors conducting the research are not being paid for including and looking after patients in the study.

8. Who has reviewed the study?

The study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Office and by the South Wales Research Ethics Committee.

9. Further information and contact details

Should you have any further queries regarding this research study, then please do not hesitate to contact me on 02920 745002 or 07746 780566. You can also contact me via e-mail on reesda@cf.ac.uk.

Thank you for considering taking part in this study.

Dr Aled Rees
Senior Lecturer in Endocrinology

You will be given a copy of this Patient Information Sheet and a signed consent form to keep.

APPENDIX 4

GIG CYMRU NHS WALES
Bwrdd Iechyd Prifysgol Caerdydd a'r Fro
Cardiff and Vale University Health Board

University Hospital of Wales
Ysbyty Athrofaol Cymru

Eich cyf/Your Ref:
Ein cyf/Our Ref: DAR/JE
Welsh Health Telephone Network 1872
Direct line/Llinell uniongyrchol
☏ 029 20742341 , 🖷 029 2074 4671

Heath Park
Cardiff CF14 4XW
Phone 029 2074 7747
Minicom 029 2074 3632

Parc Y Mynydd Bychan
Caerdydd CF14 4DW
Ffôn 029 2074 7747
Minicom 029 2074 3632

Effects of a single functional TSH receptor on bone metabolism and body composition

Patient Identification Number for this study:

PATIENT CONSENT FORM

(Version 2, December 2008)

Title of Study: TSH receptor and bone

Name of Researchers: Dr Aled Rees, Dr Anna de Lloyd

Please initial box

1. I confirm that I have read and understood the information sheet dated December 2008 (version 2) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I consent to my GP being informed of my participation in the Study. ☐
5. I consent to a pregnancy test (if indicated) . ☐

6. I agree to take part in the above study. ☐

_____	_____	
Name of patient	Date	Signature
_____	_____	
Researcher	Date	Signature
_____	_____	
Name of person taking consent (if different from researcher)	Date	Signature

1 copy for patient; 1 for researcher; 1 to be kept with hospital notes

APPENDIX 5

Participant data collection Sheet

Patient study number;

Date;

Consent; ☐

Past Medical History

Drug History

Family History (FH) Bone FH; Thyroid FH;

Social History

Occupation;

Exercise over and above the basic activities of daily living?

Example;

Average weekly alcohol intake;

Smoking History? Pack year history;

Women;-

Age of Menarche? Oligomenorrhoea? Age of Menopause?

Patient Study Number; Thyroid/ Bone Page 2

Number of pregnancies; Duration of breast feeding;

Blood Pressure;

1. 2. 3.

Height (cm);

Weight (kg);

BMI;

Waist circumference (cm); Hip circumference (cm);

Percentage body fat;

Urine Sample collected; ☐

Bloods taken?

2 x EDTA; ☐ 4-5 x SST; ☐ 1 X Fluoride Oxalate; ☐

Bloods stored; ☐ Urine Stored; ☐

DXA scan booked; ☐

APPENDIX 6: Supplementary data to Chapter 4

Total Hip MRA

This MRA evaluates against BMI as the build parameter.

N=200, R^2 = 36%

Appendix 6, Table 1: MRA evaluating total hip Z-score (assessed against BMI)

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-7.9	1.14	-10, -5.7	<0.001
Log^{10}BMI	+7	0.73	+5.6, +8.4	<0.001
Activity (1)	+0.27	0.13	+0.02, +0.5	0.04
Male (1)	-0.46	0.14	-0.7, -0.2	0.001
Log^{10} TSH mU/L	-0.25	0.34	-0.9, +0.4	0.46
T3 pmol/L	-0.35	0.12	-0.6, -0.1	0.005

Appendix 6, Table 1 provides the regression coefficients (R) for each predictor assessed in the MRA evaluating Z-score at total hip. The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

Serum corrected calcium MRA

The following parameters are *not* included in this MRA as they did not associate with calcium values: BTMs, non-sedentary lifestyle, T4 and WHR (or BMI). The corrected calcium MRA is detailed in Table 2.

N=205, R^2 = 13%

Appendix 6,Table 2: MRA evaluating calcium

Predictor	R	SE coefficient	95% C.I	p-value
Constant	2	0.09	+1.8, +2.2	<0.001
T3 (pmol/L)	+0.03	0.01	+0.01, +0.05	**0.002**
Male (1)	-0.03	0.01	-0.05, -0.01	0.006
Log^{10} TSH (mU/L)	-0.02	0.03	-0.08, +0.04	0.43
Age (years)	$+9x10^{-4}$	$4x10^{-4}$	$+1x10^{-4}$, $+2x10^{-3}$,	0.02
Log^{10} ALP (IU/L)	+0.1	0.04	+0.02, +0.2	0.01

Appendix 6, Table 2 details the regression coefficients (R) for each predictor assessed in the MRA evaluating Calcium. The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

Phosphate MRA

The phosphate MRA is summarised in Appendix 6, table 3 below.

N = 207, R^2 = 8%

Appendix 6, Table 3: MRA evaluating Phosphate

Predictor	R	SE coefficient	95% C.I	p-value
Constant	1.6	0.16	+1.24, +1.88	<0.001
T3 pmol/L	-0.01	0.02	-0.05, +0.02	0.43
Log^{10}TSH mU/L	+0.03	0.05	-0.05, +0.13	0.51
Activity (1)	-0.005	0.02	-0.04, +0.03	0.8
Male (1)	-0.04	0.02	-0.08, -0.03	0.037
Log^{10}BMI	-0.29	0.1	-0.5, -0.08	0.006
TPO (+ve)	-0.01	0.02	-0.05, +0.02	0.4

Appendix 6,Table 3 details the regression coefficients (R) for each predictor assessed in the phosphate MRA. The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus(-) or plus signs (+) indicate the direction of the associations.

Alkaline phosphatase MRAs

Appendix 6, table 4 details the alkaline phosphatase (ALP) MRA whose predictors include Z-score at TLS. ALP is log transformed to normalise the data.

N = 195, R^2 = 13%

Appendix 6, Table 4: ALP MRA (predictors include TLS Z-score)

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+1.4	0.18	+1.06, +1.77	<0.001
T3 pmol/L	-0.008	0.02	-0.05, +0.03	0.70
Log^{10}TSH mU/L	+0.04	0.05	-0.07, +0.15	0.47
TPO (+ve)	-0.04	0.02	-0.08, -0.003	0.05
Activity (1)	-0.04	0.02	-0.08, -0.03	0.035
Log^{10}BMI	+0.4	0.12	+0.12, +0.6	0.003
TLS Z-Score	-0.02	0.009	-0.04, -0.004	**0.017**

Appendix 6, Table 4 details the regression coefficients (R) for each predictor assessed in the ALP MRA (that includes TLS Z-score). The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

Appendix 6, table 5 summarises a similar ALP MRA that evaluates against TH rather than TLS Z-score. N = 197, R^2 = 15%

Appendix 6, Table 5: ALP MRA (predictors includes TH Z-score)

Predictor	R	SE coefficient	95% C.I	p-value
Constant	1.2	0.2	0.86, 1.6	<0.001
T3 pmol/L	-0.008	0.02	-0.04, +0.03	0.70
Log^{10}TSH mU/L	+0.06	0.05	-0.04, +0.16	0.26
TPO (+ve)	-0.03	0.02	-0.07, +0.006	0.10
Activity (1)	-0.05	0.02	-0.09, -0.007	0.02
Log^{10}BMI	+0.50	0.13	+0.22, +0.74	<0.001
Total hip Z-Score	-0.03	0.01	-0.05, -0.01	**0.04**

Appendix 6, Table 5 details the regression coefficients (R) for each predictor assessed in the ALP MRA (that includes Total Hip Z-score). The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

P1NP MRA

Appendix 6, table 6 summarises the P1NP MRA. P1NP was log transformed prior to analysis to normalise the data. When TH Z-score was substituted for TLS Z-score; results were similar (R -0.07, P-value 0.68). N = 185, R^2= 2%

Appendix 6, Table 6: P1NP MRA (predictors include TLS Z-score)

Predictor	R	SE coefficient	95% C.I	p-value
Constant	2.0	0.53	0.93, 3	0.00
TPO (+ve)	0.003	0.03	-0.06, +0.06	0.94
T3 (pmol/L)	0.004	0.03	-0.06, +0.06	0.9
Log^{10}TSH (mU/L)	0.03	0.09	-0.14, +0.2	0.76
Activity (1)	0.02	0.03	-0.04, +0.08	0.60
Male (1)	-0.008	0.04	-0.08, +0.06	0.82
Log^{10}BMI	-0.0007	0.2	-0.4, +0.4	1.0
Total LS Z-Score	0.008	0.02	-0.03, +0.02	0.56
Corr Ca^{2+} (mmol/L)	-0.06	0.2	-0.45, +0.34	0.8
Log^{10}ALP (IU/L)	-0.16	0.13	-0.4, +0.08	0.2
Phosphate (mmol/L)	0.002	0.12	-0.23, +0.23	1.0

Appendix 6, Table 6 details the regression coefficients (R) for each predictor variable assessed in the MRA evaluating P1NP. The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

CTX MRA

Appendix 6, Table 7 details the CTX MRA. CTX was log transformed prior to analysis to normalise the data. When TH Z-score was substituted for TLS Z-score, results were similar (R; 6 x 10-3, p-value 0.7).

N = 187, R^2= 2%

Appendix 6, Table 7: CTX MRA (predictors include TLS Z-score)

Predictor	R	SE coefficient	95% C.I	p-value
Constant	-0.12	0.58	-1.3, +1	0.84
TPO(+ve)	0.01	0.04	-0.06, +0.08	0.71
T3 (pmol/L)	0.05	0.04	-0.02, +0.11	0.17
Log^{10}TSH (mU/L)	0.01	0.1	-0.17, +0.2	0.91
Activity (1)	0.02	0.03	-0.05, +0.09	0.54
Male (1)	-0.01	0.04	-0.09, +0.07	0.80
Log^{10}BMI	-0.02	0.21	-0.4, +0.4	0.94
Total LS Z-Score	0.007	0.01	-0.02, +0.04	0.64
Corr Ca^{2+} (mmol/L)	0.21	0.21	-0.6, +0.2	0.33
log^{10}ALP	-0.09	0.14	-0.35, +0.2	0.52
Phosphate (mmol/L)	-0.05	0.13	-0.3, +0.2	0.70

Appendix 6, Table 7 details the regression coefficients (R) for each predictor variable assessed in the MRA evaluating CTX. The standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

P1NP/CTX versus alkaline phosphatase stepwise regression model

Appendix 6, Model 1: Stepwise regression model exploring R between the P1NP CTX ratio (log^{10}P1NP/log^{10}CTX) and ALP; assessed singularly and then adjusted for confounders

Step 1; Crude association

N	R	95% C.I	p-value
181	+0.45	+0.16, +0.74	0.004
Step 2; Step 1 including male sex			
181	+0.45	+0.14, +0.76	0.004
Step 3; Step 2 including age & BMI			
181	+0.54	+0.23, +0.85	0.001
Step 4; Step 3 includes TSH, T3, activity, TPO+ and TSHR Mutation status			
181	+0.55	+0.22, +0.88	0.001

Appendix 6, Model 1 details the number of subjects in the analysis (N), the correlation coefficient (R) between log^{10}P1NP/log^{10}CTX and log^{10}ALP. The 95% confidence interval (C.I.) for each analysis is provided with respective p-values. The minus (-) or plus signs (+) indicate the direction of the association.

P1NP/CTX versus DXA Z-score stepwise regression model

Appendix 6 Model 2: Stepwise regression model exploring R between the P1NP: CTX ratio (log^{10}P1NP/log^{10}CTX) and DXA Z-score (at hip and lumbar spine); assessed singularly and subsequently adjusted for confounders

	N	R	95% C.I	p-value
	Step 1; crude (adjusting for respective Z-score at that site)			
TH	183	-0.04	-1×10^{-3}, +0.07	0.06
TLS	181	-0.03	-1×10^{-3}, +0.06	0.065
	Step 2; Step 1 with adjustment for BMI and activity			
TH	183	-0.04	-0.07, +0.03	0.1
TLS	198	-0.02	-0.06, $+1 \times 10^{-3}$	0.12
	Step 3; Step 2 with adjustment for TSH, T3 and TPO+			
TH	183	-0.04	-0.08, $+5 \times 10^{-3}$	0.07
TLS	198	-0.04	-0.08, -4×10^{-3}	0.046

Appendix 6, Model 2 details the number of subjects in the analysis (N), the correlation coefficient (R) between log^{10}P1NP/log^{10}CTX and Z-score at Total Hip (TH) or Total Lumbar Spine (TLS). The 95% confidence interval (C.I.) for each step with respective p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

DXA Z-score versus male sex stepwise regression model

Appendix 6, Model 3: Stepwise regression model exploring R between DXA Z-score and male sex assessed at total lumbar spine and total hip singularly, and subsequently adjusted for confounders.

	Total Lumbar Spine				Total Hip		
N	**R**	**95% C.I.**	**p-value**	**N**	**R**	**95% C.I.**	**p-value**
	Step 1; crude (against male sex only)						
198	-0.4	(-0.8, -0.04)	0.03	200	-0.4	(-0.7, -0.07)	0.02
	Step 2; adjusted for step 2 including Log^{10}BMI and activity status						
197	-0.57	(-0.9, -0.2)	0.03	200	-0.23	(-0.8, -0.3)	<0.001
	Step 3; adjusted for step 3 including T3						
197	-0.44	(-0.8, -0.07)	0.02	200	-0.47	(-0.74, -0.2)	0.001
	Step 4; adjusted for step 3 including log^{10}TSH						
196	-0.4	(-0.75, -0.05)	0.03	200	-0.46	(-0.73, -0.2)	0.001
	Step5; adjusting for step 4 including TPO antibody positivity						
196	-0.54	(-0.9, -0.2)	0.004	200	-0.5	(-0.8, -0.2)	0.001

Appendix 6, Model 3 details the number of subjects in the analysis (N), the correlation coefficient (R) between Z-score and male at Total Lumbar Spine or Total Hip. The 95% confidence interval (C.I.) for each step with respective p-values are provided. The minus (-) or plus signs (+) indicate the direction of the association.

APPENDIX 7: Supplementary data to Chapter 5

MRA to evaluate associations with systolic BP

Appendix 7, table 1 details the systolic BP MRA against the predictors listed that include BMI as the build parameter. N=201, R^2 = 33%

Appendix 7, Table 1: MRA evaluating systolic BP with the predictors listed

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+23	19.8	-16, +62	0.24
log^{10}**BMI**	+30	12.4	+5.7, +54	0.02
Activity(1)	-1	2.2	-5.3, +3.3	0.64
Male(1)	+5	2.5	+0.1, +10	0.05
Log^{10} TSH (mU/L)	-0.5	5.8	-12, +11	0.94
T3 (pmol/L)	+11	2.1	+7, +15	**<0.001**
TPO (+ve)	-0.4	2.2	-4.7, +4	0.87
Age (years)	+0.5	0.08	+0.34, +0.66	<0.001
FOXE1 (14)	-7.8	2.2	-12, -3.5	**<0.001**

Appendix 7, Tables 1 and 2 detail the regression coefficients (R), standard errors (SE), 95% confidence interval (C.I) and p-values for each predictor variable evaluated in the systolic BP MRA (table 1) or the diastolic BP MRA (table 2). The minus (-) or plus sign (+) indicate the direction of the association.

MRA to evaluate associations with diastolic BP

Appendix 7, table 2 details the diastolic BP MRA against the predictors listed.

N=201 R^2 = 17%

Appendix 7, Table 2: MRA evaluating diastolic BP against the predictors listed

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+26.6	13.2	+0.7, +52	0.045
log^{10}BMI	+22	8.3	+5.7, +38	0.009
Activity(1)	-0.1	1.5	-3, +2.8	0.93
Male(1)	+5.7	1.7	+2.4, +9	0.001
Log^{10}TSH (mU/L)	+4.1	3.9	-3.5, +11.7	0.3
T3 (pmol/L)	+2.7	1.4	-0.04, +1.4	0.06
TPO (+ve)	+1.2	1.5	-1.7, +4.1	0.43
Age (years)	+0.1	0.06	-0.02, +0.2	0.1
FOXE1(14)	**-3.5**	1.4	-6.2, -0.8	**0.015**

MRA to evaluate associations with percentage body fat

Appendix 7, table 3 details the percentage body fat (%BF) MRA against the predictors listed.

N=205, R^2 = 28%

Appendix 7, Table 3: MRA evaluating percentage body fat.

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+27	6.5	+14, +40	<0.001
Smoking(1)	+1.8	1.2	-0.6, +4.2	0.09
Alcohol(1)	+0.06	2.1	-4, +4.1	0.92
Activity(1)	+4.9	1.1	+2.7, +7	<0.001
Male(1)	-7.9	1.3	-10.5, -5.4	<0.001
Log^{10} TSH (mU/L)	-2	3.3	-8.5, +4.5	0.5
T3 (pmol/L)	+1.9	1.2	-0.5, +4.3	0.1
T4 (pmol/L)	+0.5	0.4	-0.3, +1.3	0.3

Appendix 7, Table 3 and table 4 detail the regression coefficients (R) for each predictor variable assessed in the MRA evaluating '%BF (table 3) or WHR (table 4)'. Its corresponding standard errors (SE), 95% confidence intervals (C.I) and p-values are provided. The minus (-) or plus signs (+) indicate the direction of the associations.

MRA to evaluate associations with Waist to Hip Ratio

Appendix 7, table 4 details the waist to hip ratio (WHR) MRA against the predictors listed.

N =206, R^2 = 37%

Appendix 7, Table 4: MRA evaluating waist to hip ratio

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+0.77	0.06	+0.65, +0.89	<0.001
Smoking History (1)	+0.03	0.01	+0.01, +0.05	0.004
Alcohol (1)	-0.01	0.02	-0.05, +0.03	0.55
Activity (1)	-0.05	0.01	-0.07, -0.03	<0.001
Male (1)	+0.1	0.01	+0.08, +0.12	<0.001
Log^{10} TSH mU/L	-0.01	0.03	-0.05, +0.07	0.67
T3 pmol/L	+0.007	0.01	-0.1, +0.03	0.52
T4 pmol/L	+0.003	0.004	$-5x10^{-3}$,+0.01	0.5

MRA to evaluate associations with HOMA-IR

Appendix 7, table 5 details the HOMA-IR MRA against the predictors listed. HOMA-IR is log transformed to normalise the data.

N = 195, R^2 = 35%

Appendix 7, Table 5: MRA evaluating HOMA-IR.

Predictor	R	SE	95% C.I	p-value
Constant	-2.2	0.36	-1.5,-2.9	<0.001
WHR	+2.4	0.34	+1.7, +3	<0.001
Activity (1)	-0.2	0.05	-0.3, -0.1	<0.001
Male (1)	-0.01	0.07	-0.15, +0.1	0.2
Log^{10} TSH mU/L	-0.1	0.15	-0.4, +0.2	0.4
T3 pmol/L	+0.14	0.05	+0.04, +0.2	**0.007**

Appendix 7, Tables 5 and 6 detail the regression coefficients (R), standard errors (SE), 95% confidence intervals (C.I) and p-values for each predictor evaluated in the HOMA-IR (table 5) or LDL cholesterol (table 6) MRAs. The minus (-) or plus sign (+) indicate the direction of the association.

MRA to evaluate associations with LDL Cholesterol

Appendix 7, table 6 details the LDL cholesterol MRA against the predictors listed.
As the following predictors did not associate with LDL cholesterol; alcohol, smoking and TPO status, TSHR-M and FOXE1 PTL (14) polymorphism status, they are not included in the MRA below:-

The absence of associations with LDL cholesterol was unchanged with WHR substituted for BMI.

N = 202, R^2 = 8%

Appendix 7, Table 6: Summarises the MRA evaluating LDL cholesterol

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+0.8	1.4	-1.9, +3.5	0.6
Log^{10}BMI	+1.1	0.8	-0.5, +2.7	0.2
Activity(1)	+0.2	0.15	-0.1, +0.5	0.1
Male (1)	-0.25	0.17	-0.08, +0.6	0.14
Log^{10}TSH	+0.7	0.4	-0.08, +1.5	0.09
T3 pmol/L	+0.2	0.16	-0.1, +0.5	0.2
T4 pmol/L	-0.04	0.05	-0.06, +0.14	0.5

MRA to evaluate associations with HDL Cholesterol

Appendix 7, table 7 details the HDL cholesterol MRA against the predictors listed. HDL is square-rooted and log transformed to normalise the data.

This MRA was originally evaluated against predictors including the TFTs, TPO status and the genetic data. However, as these parameters did not associate with HDL (coefficients <0.2, p-values >0.1) they are not included in the MRA below.

N=207, R^2 = 24%

Appendix 7, Table 7: MRA evaluating HDL Cholesterol

Predictor	R	SE coefficient	95% C.I	p-value
Constant	+0.23	0.06	+0.11, +0.35	<0.001
Log^{10}BMI	-0.11	0.04	-0.2, -0.03	0.008
Activity(1)	+0.03	0.007	+0.02, +0.04	0.001
Smoking(1)	-0.01	0.008	-0.03, +0.006	0.10
Alcohol(1)	0.02	0.014	-0.007,+0.05	0.20
Male(1)	-0.05	0.008	-0.07, -0.03	<0.001

Appendix 7, Table 7 provides the regression coefficients (R), standard errors (SE), 95% confidence interval (C.I) and p-values for each predictor in the MRA assessing HDL. The minus (-) or plus signs (+) indicate the direction of the association.

MRA to evaluate associations with Total Cholesterol to HDL ratio

Appendix 7, table 8 details the Total Cholesterol to HDL ratio MRA against the predictors listed. This MRA was originally assessed against thyroid parameters including TPO status and the thyroid genetic data determined. However, as these showed no associations (R <0.03, p-values>0.1) they are not included in the MRA below. Similarly, alcohol and smoking status were assessed and showed, showed no associations and are not not included in this MRA.

The TC: HDL ratio was log transformed prior to analysis to normalise the data.

N=206, R^2 = 16%

Appendix 7, Table 8: MRA evaluating total cholesterol to HDL ratio

Predictor	R	SE Coefficient	95% C.I	p-value
Constant	-0.04	0.15	-0.3, +0.25	0.8
Log^{10}BMI	+0.35	0.1	+0.15, +0.55	<0.001
Activity(1)	-0.03	0.02	-0.07, +0.01	0.13
Male(1)	+0.06	0.02	+0.02, +0.1	0.005
T3 (pmol/L)	+0.02	0.02	-0.02, +0.06	0.27
Log^{10}TSH (mU/L)	0.03	0.05	-0.07, +0.13	0.09

Appendix 7, Table 8 provides data regarding the regression coefficients (R), standard errors (SE), 95% confidence interval (C.I) and p-values for each predictor variable evaluated within the MRA for total cholesterol to HDL ratio. The minus (-) or plus sign (+) indicate the direction of the associations.

Appendix 8: Comparative data between thyrotrophin assays

This displays the comparative analyses between the Siemens ADVIA Centeur immunoassay system and the Abbott Architect i2000SR for thyrotrophin (as a PDF document). These analyses were provided by the department of biochemistry and immunology, UHW.

TSH

Patient Comparisons

40 patient samples were analysed in duplicate for TSH using both the Siemens Centaur and Abbott i2000R to assess any bias between the two methods.

Independent variable (X) (x)			Dependent variable (Y) (y)			Mean	
Centaur			i2000R			%	Abs
1st obs	2nd obs	Mean	1st obs	2nd obs	Mean	diff	diff
0	0	0.00	0.004	0.004	0.00	200.0	0.00
0	0	0.00	0.011	0.010	0.01	200.0	0.01
0.05	0.05	0.05	0.032	0.032	0.03	43.9	0.02
0.06	0.05	0.06	0.035	0.035	0.04	44.4	0.02
0.1	0.11	0.11	0.068	0.065	0.07	44.9	0.04
0.32	0.29	0.31	0.285	0.285	0.29	6.8	0.02
0.36	0.36	0.36	0.349	0.343	0.35	4.0	0.01
0.93	0.94	0.94	0.936	0.947	0.94	0.7	0.01
1	1.13	1.07	1.054	1.063	1.06	0.6	0.01
1.17	1.18	1.18	1.063	1.072	1.07	9.6	0.11
1.11	1.2	1.16	1.069	1.088	1.08	6.9	0.08
1.23	1.32	1.28	1.205	1.212	1.21	5.4	0.07
1.27	1.28	1.28	1.310	1.296	1.30	2.2	0.03
1.66	1.68	1.67	1.375	1.421	1.40	17.7	0.27
1.47	1.54	1.51	1.491	1.483	1.49	1.2	0.02
1.81	1.8	1.81	1.507	1.540	1.52	16.9	0.28
1.88	1.74	1.81	1.636	1.616	1.63	10.7	0.18
1.73	1.92	1.83	1.574	1.678	1.63	11.5	0.20
1.99	1.89	1.94	1.659	1.726	1.69	13.6	0.25
2.1	2.12	2.11	1.955	1.969	1.96	7.3	0.15
2.48	2.25	2.37	2.085	2.113	2.10	11.9	0.27
2.37	2.29	2.33	2.114	2.160	2.14	8.6	0.19
3.09	3.34	3.22	2.542	2.592	2.57	22.4	0.65
4.32	4.74	4.53	3.953	3.926	3.94	13.9	0.59
4.51	4.63	4.57	4.229	4.300	4.26	6.9	0.31
3.7	3.81	3.76	4.301	4.310	4.31	13.7	0.55
4.88	5.64	5.26	4.538	4.588	4.56	14.2	0.70
5.95	5.92	5.94	5.510	5.523	5.52	7.3	0.42
6.73	6.33	6.53	6.080	6.139	6.11	6.7	0.42
7.45	7.64	7.55	6.713	6.664	6.69	12.0	0.86
8.74	8.57	8.66	6.931	7.019	6.98	21.5	1.68
9.37	10.34	9.86	8.202	8.130	8.17	18.7	1.69
12.4	12.81	12.61	10.182	10.434	10.31	20.0	2.30
12.47	12.35	12.41	10.779	10.683	10.73	14.5	1.68
14.66	14.95	14.81	12.787	12.810	12.80	14.5	2.01
18.29	17.3	17.80	14.388	14.690	14.54	20.1	3.26
15.75	17.69	16.72	14.528	14.888	14.71	12.8	2.01

31.2	32.06	31.63	27.296	27.131	27.21	15.0	4.42
58.08	56.37	57.23	55.332	55.436	55.38	3.3	1.84
172.51	192.41	182.46	162.104	160.485	161.29	12.3	21.17

Figure One: Comparison Plot

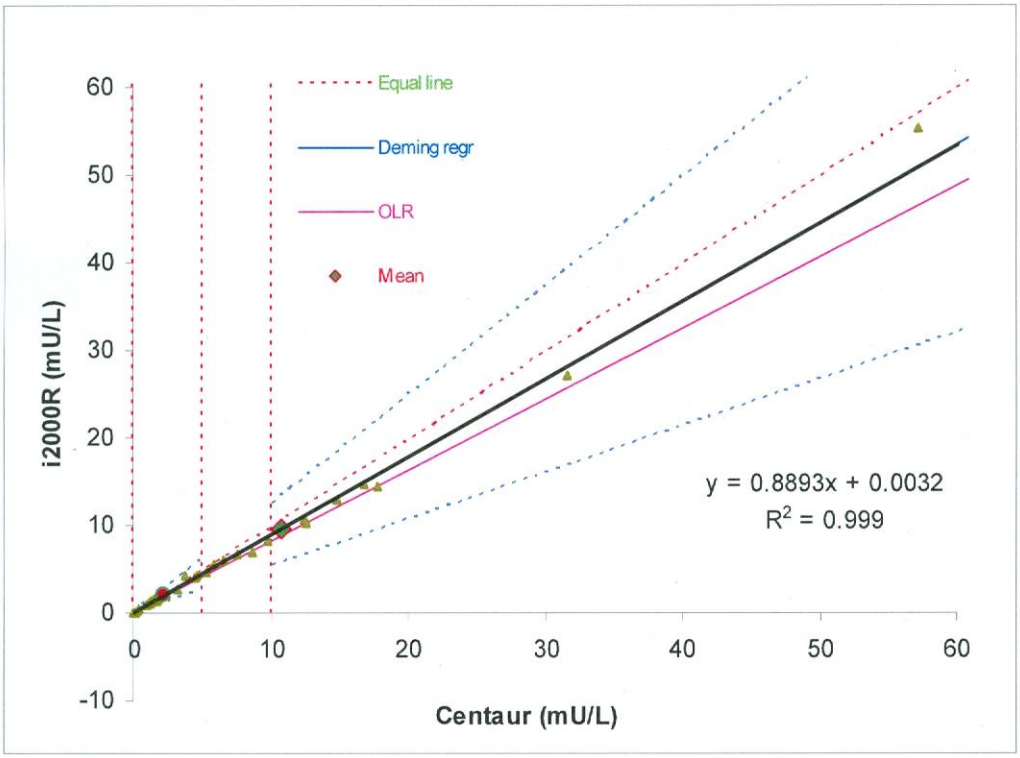


Figure Two: Plot of the absolute difference between the i2000R and the Centaur

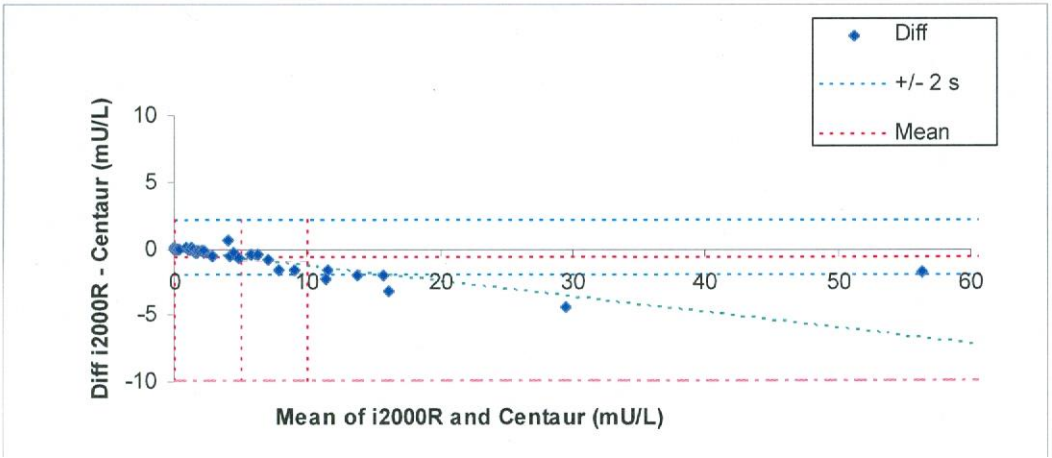
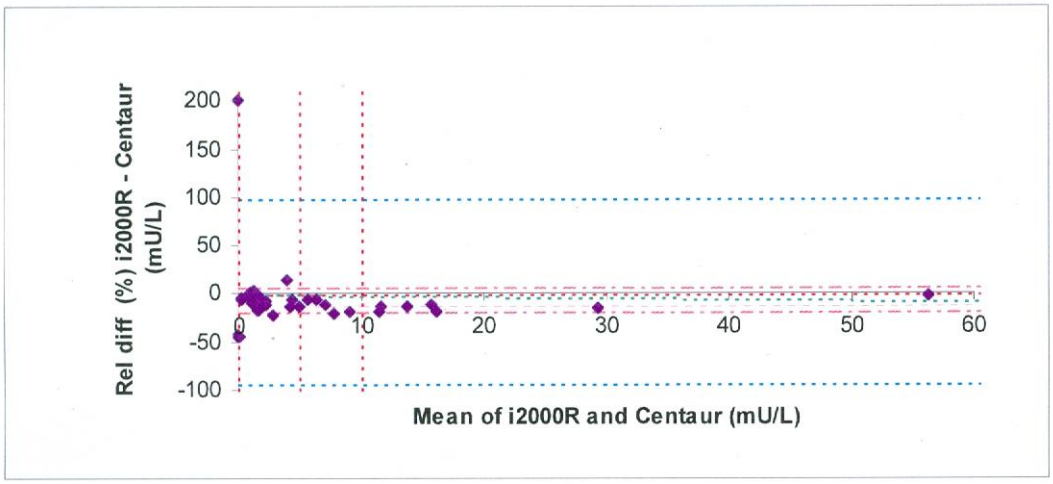


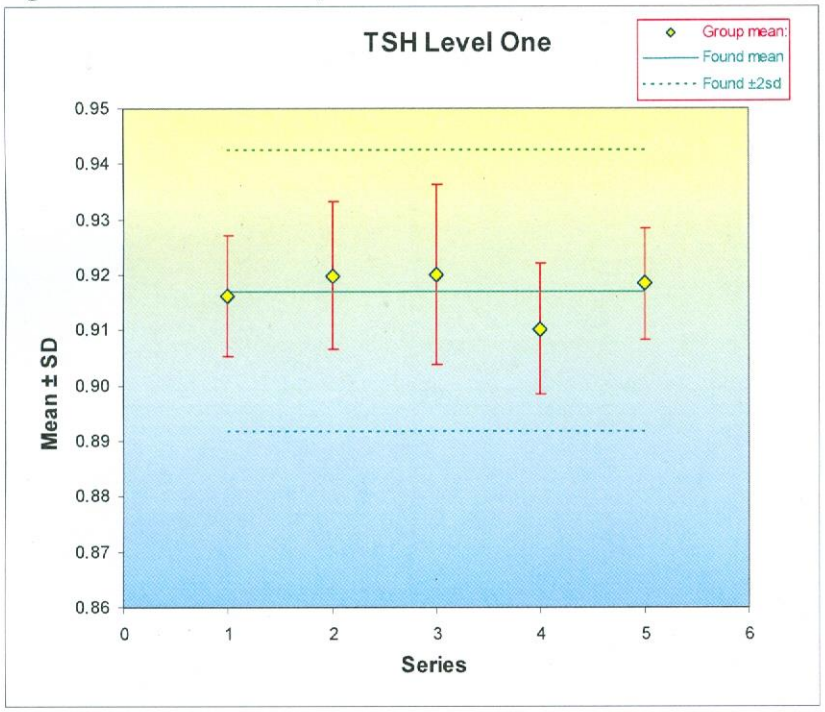
Figure Three: Plot of the relative difference between the i2000R and the Centaur.

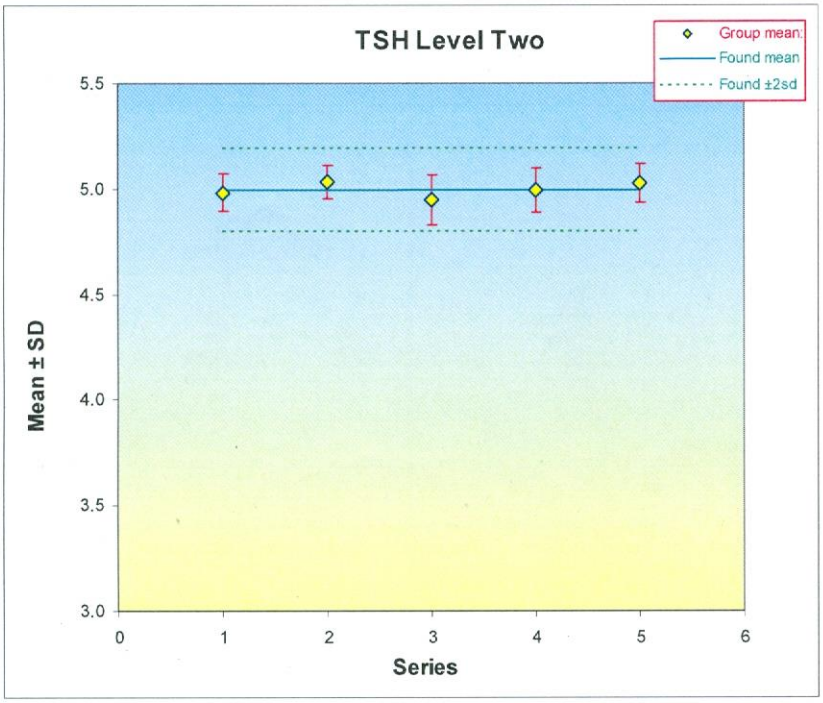


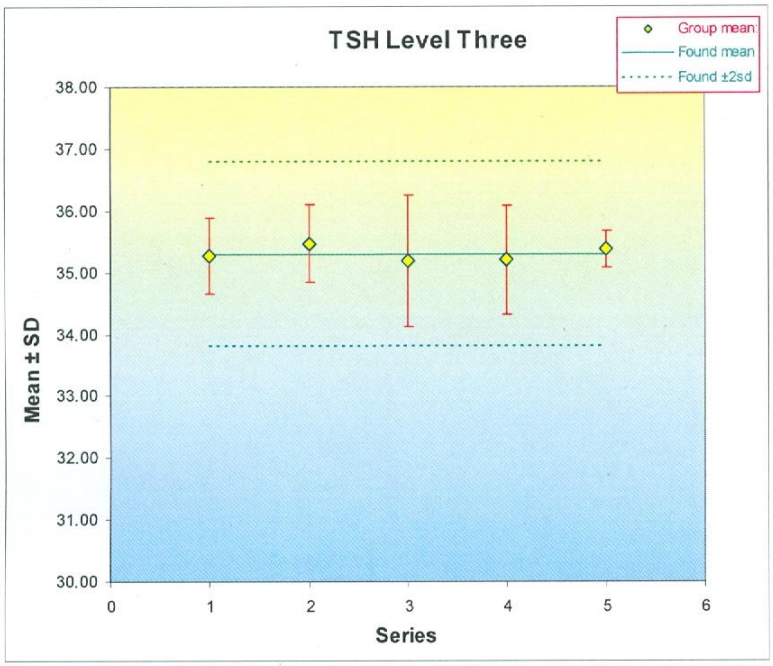
Imprecision Data

Patient samples were pooled to obtain a low, medium and high pool. These pools were then analysed over five days in replicates of five. To check for any carry-over the high pool was analysed first followed by the low pool.

Figures Four to Six: Imprecision Plots







Daily Means, SDs and CVs for Level one, two and three

Day							Mean	SD	CV
1	0.913	0.926	0.899	0.924	0.919		0.916	0.0	1.2
2	0.915	0.932	0.908	0.936	0.908		0.920	0.0	1.5
3	0.912	0.919	0.906	0.948	0.915		0.920	0.0	1.8
4	0.925	0.889	0.902	0.921	0.904		0.908	0.0	1.6
5	0.92	0.915	0.905	0.919	0.933		0.918	0.0	1.1

Day									
1	4.939	4.873	5.103	4.96	5.034		4.982	0.1	1.8
2	5.04	4.919	5.083	5.088			5.033	0.1	1.6
3	4.778	5.034	4.903	5.082	4.928		4.945	0.1	2.4
4	5.065	4.907	5.044	4.849	5.09		4.991	0.1	2.1
5	5.068	4.953	4.905	5.092	5.112		5.026	0.1	1.8

Day									
1	35.446	34.817	36.153	34.547	35.389		35.270	0.6	1.8
2	35.924	35.94	35.881	34.952	34.621		35.464	0.6	1.8
3	34.606	34.514	36.987	35.291	34.528		35.185	1.1	3.0
4	34.762	36.512	35.119	34.131	35.486		35.202	0.9	2.5
5	35.016	35.75	35.181	35.298	35.588		35.367	0.3	0.8

Overall means, standard deviations and coefficient of variations.

	Mean	SD	CV
Level One	0.917	0.013	1.4
Level Two	4.994	0.095	1.9
Level Three	35.298	0.689	2.0

EQA

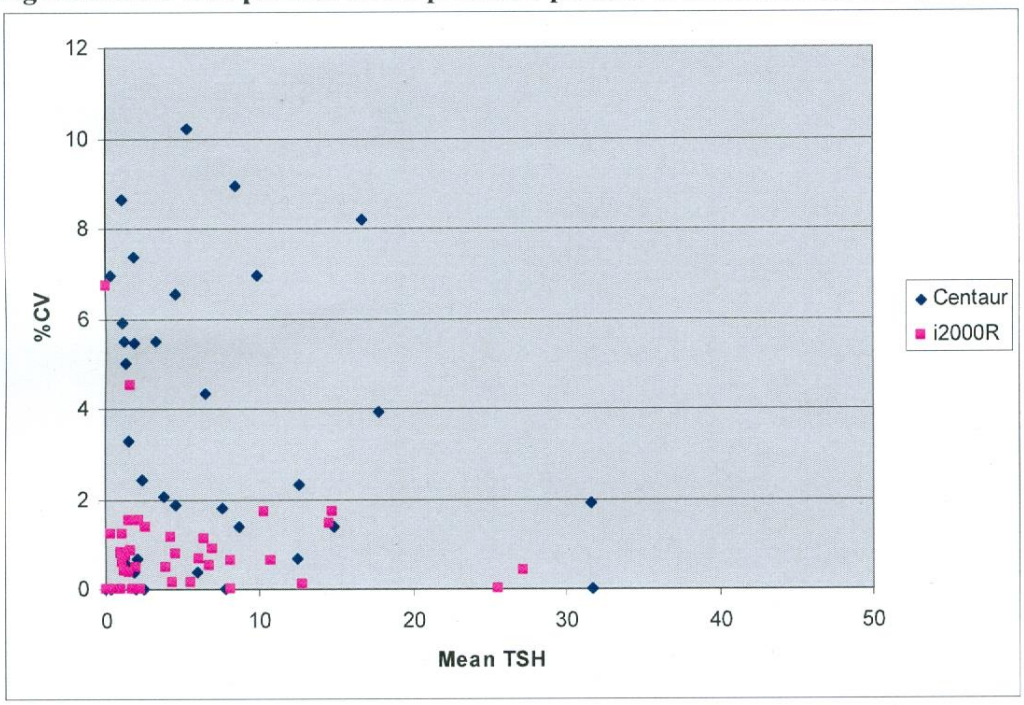
Two EQA distributions were analysed on the i2000R and compared back to the all method mean, centaur and i2000 method means.

Specimen No.	ALTM	Architect Method Mean	Architect Result 1	Centaur Method Mean	Centaur Reported result
334A	1.33	1.23	1.17	1.36	1.25
334B	3.21	3.13	3.12	3.2	3.13
334C	5.1	4.99	5.03	5.13	4.68
334D	6.91	6.76	6.54	7.03	6.57
334E	8.8	8.6	8.59	9.02	8.03
336A	1.49	1.32	1.29	1.55	1.44
336B	2.5	2.33	2.36	2.54	2.29
336C	3.57	3.38	3.48	3.62	3.19
336D	6.79	6.42	6.43	7.05	6.19
336E	11.98	11.33	11.41	12.45	11.07

CV Profiles

CVs were calculated across the analytical range using the patient comparison duplicates.

Figure Seven: Comparison of the precision profiles of the i2000 and the Centaur.



Instrument Dilution Check

One patient sample with a high concentration of TSH was diluted both manually and onboard the i2000 analyser.

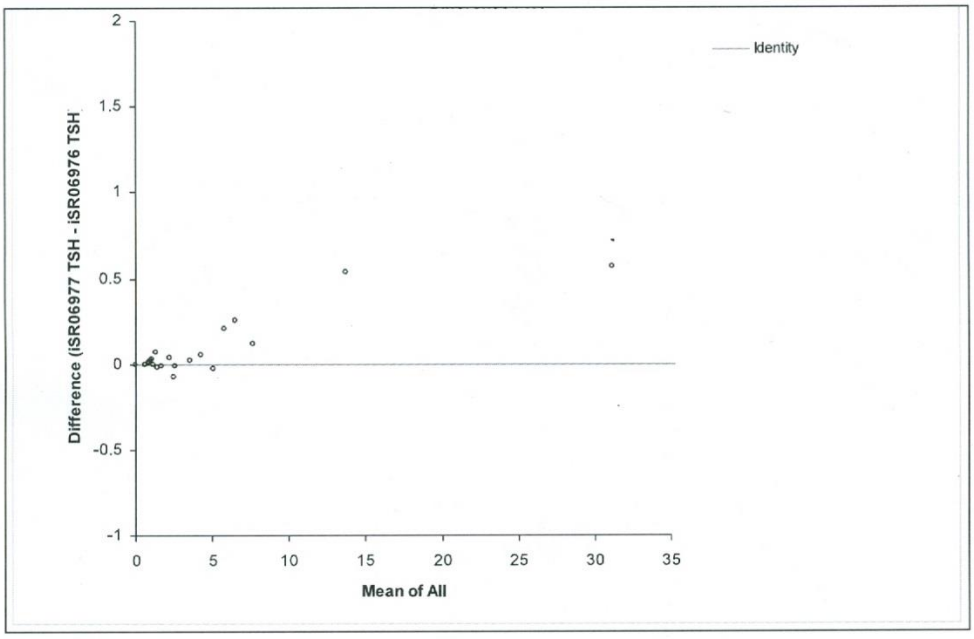
Manual Dilution	Manual Dilution	Mean	On-board	On-board	Mean	% diff
146.3	147.8	*147.1*	157.5	159.7	*158.6*	7.8

Silver Evaluation

Comparison of the i2000R (gold instrument) to further analysers (silver instrument) installed in the laboratory by analysing 25 patient samples on both analysers.

TSH.1	TSH.2
0.021	0.021
0.619	0.615
0.912	0.929
0.921	0.928
1.008	1.030
1.081	1.114
1.134	1.130
1.283	1.350
1.442	1.420
1.728	1.716
2.198	2.232
2.530	2.456
2.603	2.589
3.580	3.598
4.273	4.327
5.124	5.099
5.647	5.851
6.414	6.672
7.582	7.696
13.486	14.022
30.870	31.441
165.015	171.487

Figure Eight: Difference plot between the two analysers



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0.619	0.615
0.912	0.929
0.921	0.928
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1.081	1.114
1.134	1.130
1.283	1.350
1.442	1.420
1.728	1.716
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4.273	4.327
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5.647	5.851
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30.870	31.441
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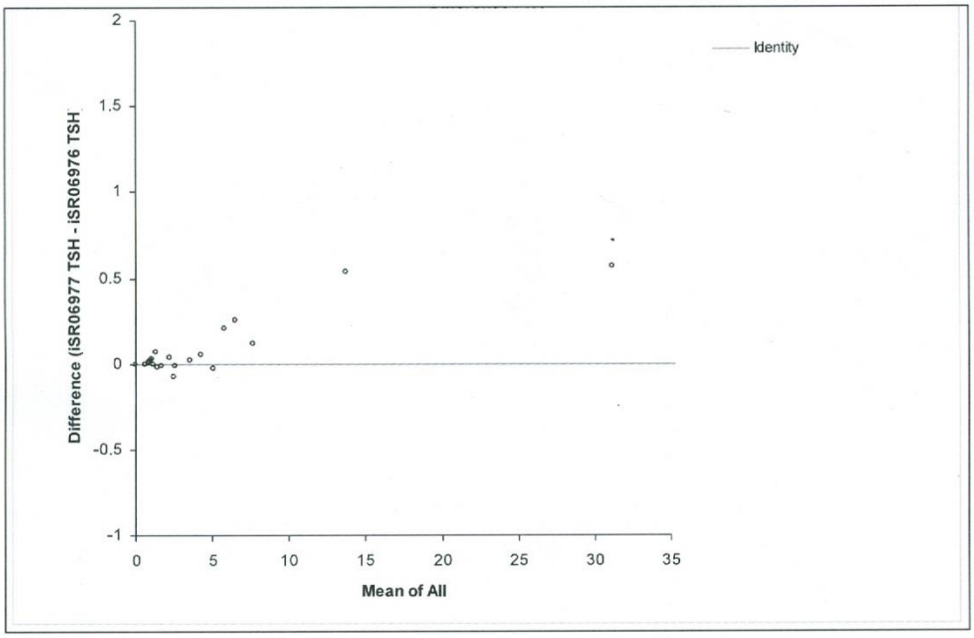

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