The prevalence and natural history of normocalcaemic hyperparathyroidism and hypoparathyroidism

By

Marian Schini

A thesis submitted for the degree of Doctor of Philosophy

The University of Sheffield

Faculty of Medicine, Dentistry & Health

Department of Oncology and Metabolism

January 2020

To my family and friends
who have accompanied me in this journey

Section 1: Acknowledgments

I would like to thank Professor Richard Eastell for his continuous support, supervision and help. You have been more than a supervisor; you have been a mentor, a colleague, a friend. I would also like to thank my second supervisor Dr Jennifer Walsh. Coming to you always resolved issues in a very efficient way. You have always been supportive and inspiring. A special thank you to my previous supervisor, Gregory Kaltsas for giving me the opportunity to come to Sheffield in the first place, and to Professor John Newell Price for accepting me.

This project would not have been possible if it was not for my funders. A special thank you to CIMA and Osteoporosis 2000 for their financial support.

I would like to send my thanks to all the members of the Academic Unit of Bone Metabolism who have accompanied me throughout this journey. Gill, thank you for your constant help and morning chats. Margaret, thank you for keeping me company in the office and for providing me information on scanning and ethical applications. Fatma, thank you for your support with the laboratory data on several occasions and for teaching me about Sheffield. Special thanks to the Metabolic Bone Unit for their help and support. Diane, thank you for the information on DXA scanning. I would like to thank Dr Nicola Peel for all her input, help and guidance with clinical and research issues. Thank you for making me a better clinician in the field. I would like to extend this appreciation to other consultants of the unit, Eugene, Mary and Kate. A really special thank you to Richard Jacques. You helped me tremendously throughout this project, with your analyses, support and discussions. A big thank you to the statisticians in MASH for their support. I would like to express my appreciation to the people in Sheffield Teaching Hospitals lab, especially Eleanor Oakes for her constant

support. Finally, Thomas Butterfield, this project would had not been possible if it was not for you. Sorry for asking for the “longest and most demanding” databases.

Of course, this journey would had not been possible without the support from my friends. Even though you are far away, your love keeps me going and makes me stronger. I have been fortunate to have good friends in several places. In Cyprus, special thanks to Eleni, Efi, Lefkios, Kyriaki, Melios, Georgia, Magda, Maria, Victor and partners. Your texts and calls kept me going. Thank you to my friends and colleagues in Greece, Michalis, Ioanna, Mety and Symeon. A big thank you to Christos for keeping me going, not just now, but for the past years. Sheffield has been my home for the last few years. I would like to thank the friends I made here for their love and support. Eleni, Costas, Louis, Valentina, Joy and Claire, you have all been great. Tatiane Vilaca, a special thank you to you for being next to me through all these ups and downs of a PhD journey.

The completion of my thesis would not had been possible without the constant support and help from Luis and his family. Luis, I cannot even express my gratitude in words for the inspiration, for constantly pushing me to achieve more and for making me better.

Finally, nothing of this would have even started if it was not for my family. You have been on my side throughout my whole life and made me who I am today. I progressed and succeeded only knowing that you are there for me. I love you all.

Section 2: Declaration

This thesis is submitted in the form of the Alternative Format Thesis which allows composing thesis from academic manuscripts (published or unpublished), alongside with the traditional thesis sections.

I hereby certify that this thesis includes work formatted to be submitted in scientific journals of which I am a joint author. I have included a written statement from co-authors, and my supervisor Professor Richard Eastell. The contribution statements are included before each relevant manuscript.

Section 3: Conference presentations and prizes

- Normocalcaemic Hyperparathyroidism: Study of the prevalence and natural history in a United Kingdom referral population

  National Osteoporosis Society Conference, December 2018, Birmingham, UK

  Premier poster award

- Normocalcaemic Hypoparathyroidism (NHYPO): Study of the prevalence and natural history in a United Kingdom referral population

  9th Annual Mellanby Research Day, November 2018, Sheffield, UK

  First poster award

- Normocalcaemic Hyperparathyroidism: Study of the prevalence and natural history in a United Kingdom referral population

  ASBMR 2018 Annual Meeting, September 2018, Montréal, Québec, Canada

- Normocalcaemic Hyperparathyroidism: Prevalence In a United Kingdom referral population

  European Calcified Tissue Society (ECTS) Congress, May 2017, Salzburg, Austria

Section 4: Abstract

Normocalcaemic hyperparathyroidism (NPHPT) is characterised by persistently normal calcium levels, elevated PTH values on at least two consecutive measurements, after excluding other causes of secondary hyperparathyroidism. The prevalence of the disease in the literature varies significantly due to various definitions used; it is reported to be between 0.1 and 8.9%. The data on the natural history of this disease are sparse and inconclusive. Normocalcaemic hypoparathyroidism (NHYPO) is characterised by persistently low levels of parathyroid hormone (PTH) with normal levels of calcium. There is little in current literature on this disease, with only two studies published on its prevalence whilst its natural history remains relatively unknown.

The aims of this study were: to identify the prevalence of NPHPT in a UK referral population using the international criteria and study the natural history of this disorder and the variability of serum calcium; to compare the variability of calcium in NPHPT and PHPT; to identify the prevalence of NHYPO and to study the natural history of the disorder and the variability of serum calcium.

The prevalence of NPHPT in our UK referral population was found to be low. NPHPT patients often have episodes of hypercalcemia, so they probably suffer from PHPT. The variability of NPHPT and PHPT patients is similar.

The prevalence of NHYPO calculated from this UK referral population is lower when compared to results from previous studies. NHYPO patients often have episodes of hypocalcaemia with some cases having no apparent reason for calcium levels below the reference range. The next steps are to find out the cause of this biochemical abnormality and the consequences, if any.

Section 5: Abbreviations

7-DHC	7-dehydroxycholesterol
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
APOA	Apolipoprotein A
APOB	Apolipoprotein B
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUBM	Academic unit of bone metabolism
BA	Bone area
BCG	Bromcresol green
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
BSA	Body surface area
BTM	Bone turnover markers
CaMK	Calmodulin-dependent protein kinase
CaSR	Calcium-sensing receptor
CCCR	Creatinine clearance ratio
CGPS	Copenhagen General Population Study
CHIAG	Community Health Index Advisory Group
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration
CLIA	Competitive chemiluminescence immunoassay

CLSI	Clinical & Laboratory Standards Institute
cQCT	Central quantitative computed tomography
CrCl	Creatinine clearance
CRP	C reactive protein
CT	Computerised tomography
CTX	Collagen type 1 cross-linked C-telopeptide
CV	Coefficient of variation
CVA	Cerebrovascular accident
DAG	Diacylglycerol
DBP	Vitamin D binding protein
DCH	Danish Diet, Cancer, and Health Study
DHS	Dallas Heart Study
DM	Diabetes mellitus
DOPS	Danish Osteoporosis Prevention Study
DXA	Dual energy X-ray absorptiometry
ECF	Extracellular fluid
ECG	Electrocardiogram
ECLIA	Electrochemiluminescence immunoassay
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
EQA	External quality assurance
EQAS	External quality assessment schemes
ESR	Erythrocyte sedimentation rate
FDA	American Food and Drug Administration
FHH	Familial hypocalciuric hypercalcaemia
FNA	Fine needle aspiration
FRAS	Fracture risk assessment service

FSH	Follicle-stimulating hormone
FT	Foundation trust
GDPR	General Data Protection Regulation
GFR	Glomerular Filtration Rate
GGT	Gamma-glutamyl transpeptidase
GP	General practitioner
GPCR	G-protein coupled receptor
HADS	Hospital Anxiety and Depression Scale
HAMA	human anti-mouse antibodies
HCL	Hydrochloric acid
HDL	High density lipoprotein
HH	Hyperparathyroid hypercalcaemia
HOMA-IR	Homeostatic model assessment for insulin resistance
HR	Hazard ratio
HTA	Human Tissue Authority
iCa	Ionised calcium
IDMS	Isotope-dilution mass spectrometry
IGF-1	Insulin growth factor 1
IGT	Impaired glucose tolerance
IH	Idiopathic hypercalciuria
IHD	Ischaemic heart disease
IOF	International Osteoporosis Foundation
IOM	US Institutes of Medicine
IP_3	Inositol 1,4,5-trisphosphate
IQR	Interquartile range
IRMA	Immunoradiometric assay
ISCD	International Society for Clinical Densitometry

IT	Information Technology
JCTLM	Joint Committee for Traceability in Laboratory Medicine
KDIGO	Kidney Disease Improving Global Outcomes
LDL	Low density lipoprotein
LH	Luteinizing hormone
LPA	Lipoprotein A
LRP6	Low-density lipoprotein receptor related protein 6
LSC	Least significant change
MBC	Metabolic bone centre
MD	Mahalanobis distance
MDRD	Modification of diet in renal disease
MEN	Multiple endocrine neoplasia
MIP	Minimally invasive parathyroidectomy
MREC	Multi-Centre Research Ethics Committee
MRI	Magnetic resonance imaging
MrOS	Osteoporotic Fractures in Men Study
MTA	Material Transfer Agreement
MVE	Minimum volume ellipsoid estimator
NGH	Northern General hospital
NHANES III	National Health and Nutrition Examination Survey
NHS	National health service
NPHPT	Normocalcaemic hyperparathyroidism
NHYPO	Normocalcaemic hypoparathyroidism
NICE	National Institute for Health and Care Excellence
NIGB	National Information Governance Board for Health & Social Care
NS	Not significant
o-CPC	o-cresolphthalein complexone

OGTT	Oral glucose tolerance test
OGTT	Glucose tolerance test
OPG	Osteoprotegerin
OPUS	Osteoporosis and Ultrasound Study
P1NP	Procollagen type 1 N-terminal propeptide
PCA	Principal component analysis
PHPT	Primary hyperparathyroidism
PIAG	Patient Information Advisory Group
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PPI	Proton pump inhibitors
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related peptide
QC	Quality control
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor κB ligand
RF	Rheumatoid factor
RHH	Royal Hallamshire hospital
RIA	Radioimmunoassay
RLU	Relative light units
ROI	Region of interest
ScHARR	School of Health and Related Research
SCr	Serum creatinine

SD	Standard deviation
SE	Standard error
SF-36	Short Form 36
SHBG	Sex hormone-binding globulin
SNP	Nucleotide polymorphism
SOP	Standard Operating Procedures
STH	Sheffield Teaching Hospitals
S_w	Within-subject standard deviation
tCa	Total calcium
TRACP 5b	Tartrate-resistant acid phosphatase 5b
TRCa	Tubular reabsorption of calcium
TRPV6	Transient receptor potential cation channel
TSH	Thyroid-stimulating hormone
UCCR	24hour urine calcium to creatinine ratio
UV-B	Ultraviolet B
VDBP	Vitamin D binding protein
VFA	Vertebral fracture assessment
VLDL	Very low density lipoprotein
WHO	World Health Organization

Section 6: Overview of the thesis chapters

The alternative format for writing a thesis was applied in this document, as approved by the University of Sheffield in 2019. This implies that some information might be mentioned more than once, as the final results are presented in a scientific manuscript format eligible for publication. The outline of this thesis is as follows.

Chapter 1: Introduction

The introduction is giving some background information on calcium metabolism and parathyroid disorders related to hypercalcaemia (primary hyperparathyroidism) and hypocalcaemia (hypoparathyroidism). Endocrine disorders are often characterised by a clinical and a subclinical form. Following a similar pattern, two disorders related to calcium and the parathyroid glands were recently described in literature. Normocalcaemic hyperparathyroidism (NPHPT) is a disorder of calcium metabolism which is characterised by persistently normal calcium levels and consistently elevated parathyroid hormone (PTH) values. A pathophysiological counterpart to NPHPT is normocalcaemic hypoparathyroidism (NHYPO), which is characterised by normal levels of calcium with low levels of PTH. A literature review is presented and the gap in literature is identified.

The introduction is followed by the rationale of the study and the aims of the thesis.

Chapter 2: Methods and method development

The main data for this thesis were retrieved from a tertiary metabolic bone referral centre. The methods section gives some information on the centre and describes the

theory behind the different methods used in the studies. This is then followed by a method development section, where the results from initial analyses are presented. This is done in order to describe the thought process followed before reaching the final analysis presented in the next chapters.

Chapter 3: Metabolic Bone Centre studies

NPHPT and NHYPO. Prevalence and natural history

This chapter contains the final results of the analyses performed using data from the tertiary referral centre. The format used is a scientific paper format as indicated in the alternative thesis format guidance by the University of Sheffield. Both the manuscripts included were formatted for submission in peer reviewed journals.

Some extra analyses performed to complement the chapter are added in the end.

Chapter 4: UK Biobank studies

As the main part of the study was performed in a referral centre, a population-based database was used to further study the variability of calcium and compare the findings with the ones presented in Chapter 3.

Chapter 5: Final discussion and future plans

Although each chapter contains a summary of the findings and discussion, a final one is included in the end of the thesis to also introduce future research plans

Appendix

A list of tables on published studies performed on normocalcaemic hyperparathyroidism is added to supplement the introduction.

These are followed by the intermediate analyses performed before the final one presented in Chapter 3.

The bibliography used in this thesis is also attached in this section.

Supplementary material

These include the codes done in R and are attached in a CD.

Section 7: Table of contents

Section 8: Table of tables

Section 9: Table of figures

Chapter 1: Introduction

Section 1: The physiology of calcium

Section 2: Parathyroid gland disorders

Section 3: Rationale of the study and aims

Section 1: The physiology of calcium

Calcium is an important mineral in the human body; its regulation within tight normal limits is of great importance. Calcium is involved in several processes like cell division and adhesion, secretion of proteins from cells, muscle and nerve function, glycogen metabolism and, it also acts as a co-factor during coagulation procedures. The human body contains approximately 1000g of calcium, mainly located in the bones as hydroxyapatite (99%); the remaining 1% is located extracellularly, in the blood and soft tissues. Calcium can be found in three different forms in the blood. Approximately 45% of the total calcium (tCa) is ionised (iCa) and this is the biologically active form, 45% is bound to plasma proteins, mainly albumin, and 10% forms complexes with citrate or PO4 ions (Favus & Goltzman, 2013). Because of this affinity of calcium with proteins, total calcium levels can vary according to the albumin concentrations. Therefore, in the case of dehydration or haemoconcentration during venepuncture, where albumin increases, there are false elevations of the total calcium. To avoid this, the value of calcium is adjusted for the albumin concentration using an equation based on their regression. Changes in the blood pH can also affect calcium levels; ionised calcium increases in acidosis due to reduced binding by albumin (Barth et al, 1996; Vautour & Goltzman, 2018).

Parathyroid hormone (PTH) is the main regulator of calcium homeostasis and it is secreted by the chief cells of the parathyroid glands (Favus & Goltzman, 2013).

1.1.1 Parathyroid glands

Developmentally, the parathyroid glands derive from the pharyngeal pouches. These are transient structures which differentiate from the foregut endoderm; the superior

glands derive from the fourth ones and the thymus and inferior glands from the third ones. They first appear in the fifth week of gestation. Their development is associated with a conservative hierarchy of genes (Rubin & Levine, 2013).

The first reference to the parathyroid glands in the literature was made in 1852 by Sir Richard Owen, a Conservator of the Hunterian Collection of the Royal College of Surgeons. He performed a necroscopy of an Indian rhinoceros and reported “a small compact yellow glandular body, attached to the thyroid at the point where the veins emerge”. The full credit for the description and naming of the glands (glandulae parathyroidae) belongs to Ivar Sandström, a Swedish anatomist, who first noticed the glands in a dog and then identified and described them in several species (rabbits, cats, horses and humans). He found “on both sides at the inferior border of the thyroid gland an organ of the size of a small pea”. He published his work in 1880 in a local journal, but it was not really noticed for some years. The importance of these glands was recognised in 1891 by Eugene Glay, professor of physiology in Paris, who proved that it was their removal after a thyroidectomy that caused the fatal seizures already described as “tetany”. Two Italian researchers, Giulio Vassale and Francesco Generali showed in 1896, that tetany was caused by parathyroidectomy alone, leaving the thyroid gland intact. The assumption was that parathyroid glands were responsible for the removal of some toxins from the body and that the tetany was caused by intoxication. The toxin suggested was methyl guanidine. This theory was kept until several years later, when William MacCollum and Carl Voegtlin working at Johns Hopkins Medical first suggested in 1909 that it was the parathyroid glands that were responsible for the regulation of calcium and that tetany was caused by calcium deficiency and could be prevented by the administration of calcium (Eknoyan, 1995; Johansson, 2015).

1.1.2 Parathyroid hormone (PTH)

The identification of the hormone responsible for the effects on calcium was performed by two independent researchers, Adolph Hanson in 1923 and James Collip in 1925. They both used similar methods to extract the hormone, but the credit was given to Collip, a person already known for his contribution to the discovery of insulin. He used hot hydrochloric acid to retrieve parathyroid extracts and could treat the tetany caused by parathyroidectomy by giving these extracts subcutaneously to dogs. This resulted in the normalisation of calcium. The researcher established the parathyroid glands as endocrine organs, producing parathyroid hormone. However, it was not until several years later (1959), that Aurbach, Rasmussen and Craig managed to purify PTH using improved procedures (Eknoyan, 1995; Gardella et al, 2010; Johansson, 2015)

Parathyroid hormone is an 84-amino acid polypeptide whose gene is located on the short arm of chromosome 11p15. It is produced by the chief cells of the parathyroid glands and has a molecular weight of 9400 daltons. The amino acid sequence of PTH is extensively conserved amongst mammalians, especially in the N-terminal where 32 out of 38 residues are conserved. This part of the hormone is necessary and sufficient for the binding and activation of the PTH receptor and for the mineral ion homeostasis. The part with the greatest variation is the middle one (amino acids 39-52), which has limited importance. There is also high homology in the C terminal (Gardella et al, 2010; Murray et al, 2005).

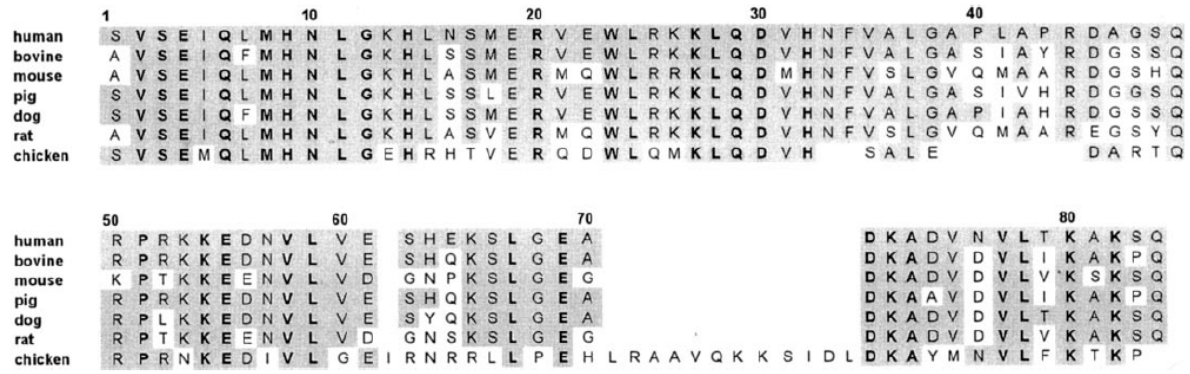


Figure 1-1: Amino acid sequencing of PTH in different species.

Shading indicates that the residues are conserved in other species, while the bold letters indicate that the residues are conserved in all species. The amino acids are G: Glycine, P: Proline, A: Alanine, V: Valine, L: Leucine, I: Isoleucine, M: Methionine, C: Cysteine, F: Phenylalanine, Y: Tyrosine, W: Tryptophan, H: Histidine, K: Lysine, R: Arginine, Q: Glutamine, N: Asparagine, E: Glutamic Acid , D: Aspartic Acid, S: Serine , T: Threonine. From: Parathyroid Hormone Secretion and Action: Evidence for Discrete Receptors for the Carboxyl Terminal Region and Related Biological Actions of Carboxyl-Terminal Ligands, Endocr Rev. 2005;26(1):78-113. doi:10.1210/er.2003-0024, Endocr Rev | Copyright © 2005 by The Endocrine Society

There is a 141 amino acid protein, called PTH-related protein (PTHrP), which shares homology with PTH. This is encoded in chromosome 12. It was first thought to just be a mediator of hypercalcaemia in malignancy, but nowadays, it is believed that it has a role in the development of the skeleton and that it regulates calcium through pregnancy and lactation. These two proteins have eight out of the first 13 residuals of the amino acid sequence identical. That explains the shared actions on the PTH receptor (Vautour & Goltzman, 2018).

1.1.2.1 Synthesis

PTH is synthetized as a pre-pro-PTH molecule with a 25-amino acid sequence in the N terminal, followed by the 90-amino acid sequence of pro-PTH. This is the first molecule formed form the translation of the mRNA in the ribosomes. This 25-amino acid sequence is rich in hydrophobic amino acids, which is considered to be important for the binding to the endoplasmic reticulum and for entering the cisternal space. The pre sequence is removed and the pro-PTH is transported to the Golgi network where the pro-sequence (amino acids -6 to -1) is cleaved with the enzyme furin. The mature form (PTH 1-84) is then packed into secretory vesicles within 20-30 minutes from the beginning of the process. The content of these vesicles can be stored, degraded or secreted in the bloodstream. Calcium and vitamin D do not seem to affect the synthesis of the hormone, but can adapt the degradation of the hormone in the vesicles. Proteases cathepsin B and H are involved in the degradation of the hormone and generation of the carboxyl fragments (Cohn & MacGregor, 1981; Gardella et al, 2010).

1.1.2.2 PTH receptors

PTH mainly binds on the PTHR1 receptor (also called PTH/PTHrP common receptor), which belongs to the G protein receptor superfamily. This receptor can be found in the surfaces of bone cells like osteoblasts and osteocytes and on the tubular cells in the kidney. The gene encoding the receptor is located at chromosome 3 (Silva & Bilezikian, 2015). It is formed by three parts, an extracellular part of approximately 165 amino acids, a transmembrane part with seven membrane helices and loops connecting them and, an intracellular part of 130 amino acids (Figure 1-2). This

receptor is highly expressed in the kidney and bones and can be stimulated by both PTH and PTH related peptide (PTHrP) by the “two-site” model of binding (Figure 1-3). After the receptor is activated, it acts through different second messenger pathways; through the GSa subunit, it stimulates adenyl cyclase, which in turn, catalyses the conversion of ATP to cyclic AMP and thus activates protein kinase A (PKA). Moreover, it can act through the Gq subunit and activate phospholipase C (PLC), a membrane bound enzyme which, by hydrolysing phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2), leads to the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The former moves into the cytoplasm and by stimulating receptors on the endoplasmic reticulum, it stimulates calcium mobilization. DAG on the other hand, is membrane-bound and is responsible for the activation of protein kinase C (PKC). Another possible action of PTH is the translocation of β-arrestins to the cell membrane, proteins known for their effect to regulate G receptors. These proteins then downregulate the cAMP activation and stimulate the mitogen-activated protein kinase (extracellular signal-regulated kinase [ERK1/2]) signaling cascade (Gardella & Vilardaga, 2015; Murray et al, 2005; Silva & Bilezikian, 2015).

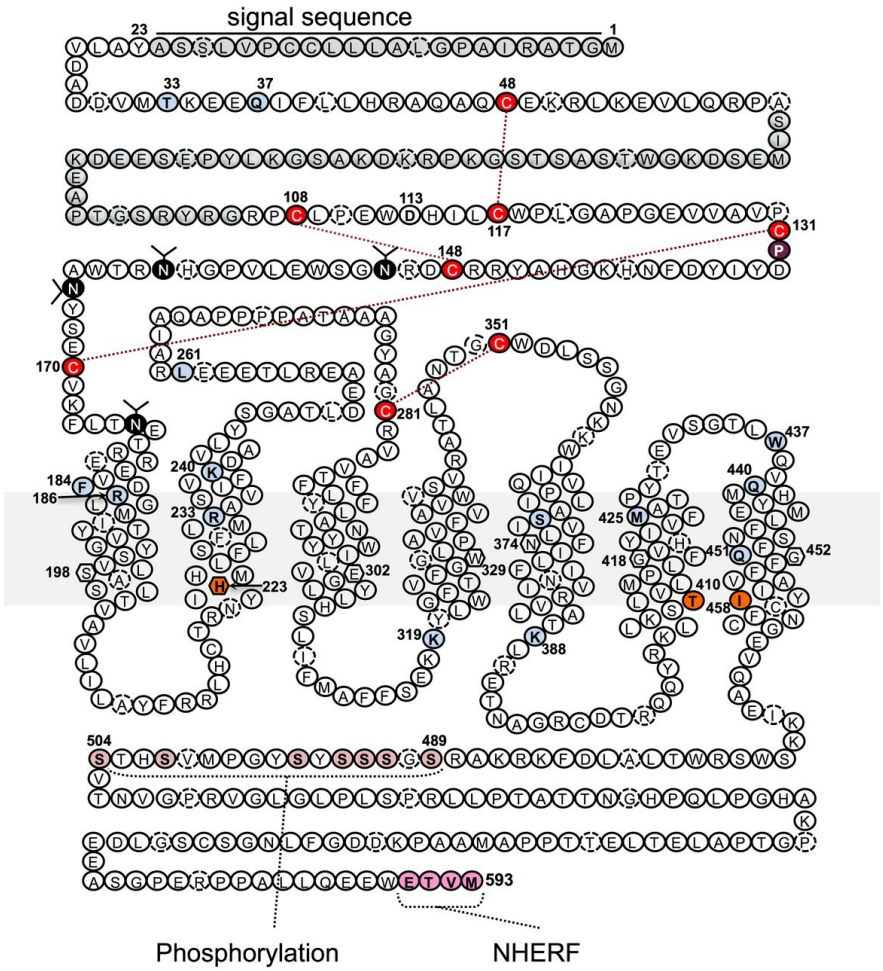


Figure 1-2: The PTH receptor type 1.

The receptor consists of 593 amino acids, with a large extracellular part of about 160 amino acids (the initial N-terminal is removed), a transmembrane part of seven helices with loops connecting and an intracellular part. Reprinted from Gardella TJ, Vilardaga JP. International Union of Basic and Clinical Pharmacology. XCIII. The parathyroid hormone receptors--family B G protein-coupled receptors. Pharmacol Rev 2015; 67:310-337. Permission from the American Society for Pharmacology & Experimental Therapeutics

A second receptor has been identified, called PTH2 receptor (PTH2R). This receptor is not located at the kidneys or bones but in other tissues like hypothalamus, heart, somatostatin cells at the pancreas, thyroid parafollicular cells, vascular endothelium,

gastrointestinal tract, etc. This receptor is only activated by PTH, not by PTHrP; however, its endogenous ligand is not PTH, but a 39-amino acid polypeptide called tuberoinfundibular peptide 39 (TIP39). This peptide was found to have functions in the neuroendocrine system (increase production of hormones) and spermatogenesis. More recently, a third type of receptor (PTH3R) has been discovered in animals but not in mammals and its role is uncertain (Gardella & Vilardaga, 2015; Murray et al, 2005).

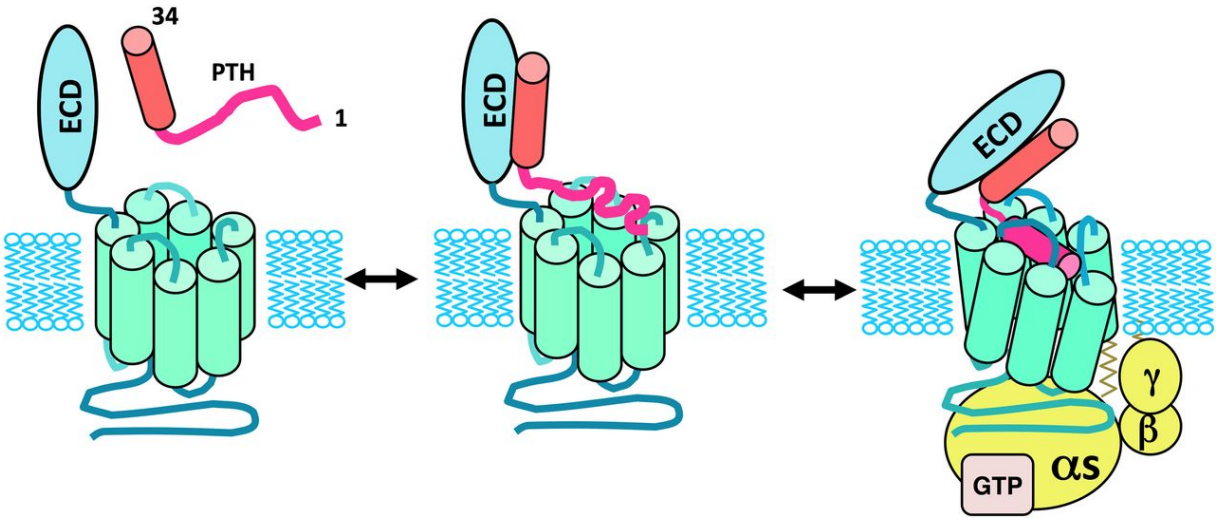


Figure 1-3: The "two-site" model of PTH/PTHR1 interaction.

The 15-34 terminal of PTH binds to the extracellular part of the receptor (ECD) and then the N-terminal binds to the transmembrane. This leads to changes in the receptor and hence coupling of the G proteins. Reprinted from Gardella TJ, Vilardaga JP. International Union of Basic and Clinical Pharmacology. XCIII. The parathyroid hormone receptors--family B G protein-coupled receptors. Pharmacol Rev 2015; 67:310-337. Permission from the American Society for Pharmacology & Experimental Therapeutics

1.1.2.3 Actions

1.1.2.3.1 Skeleton: catabolic and anabolic actions

PTH has both catabolic and anabolic effects on the skeleton. Its effects are direct on osteoblasts and osteocytes and indirect on osteoclasts. The effects depend on the duration and periodicity of exposure. When there is continuous exposure to high levels of PTH, the effect is catabolic, therefore increasing bone resorption. This effect is mediated through the OPG–RANKL–RANK pathway. PTH acts on osteoblasts and stimulates the production of the receptor activator of nuclear factor κB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG). RANKL binds to the receptor activator of nuclear factor-kappa B (RANK) found on osteoclasts and their hematopoietic precursors. The differentiation and survival of the precursors is promoted and so is the stimulation of the fully formed osteoclast. OPG is able to inhibit the bone resorption by binding to RANK and the balance between the concentration of OPG and RANKL regulates the degree of bone resorption. The bone resorption releases minerals including calcium from the bone (Favus & Goltzman, 2013; Gardella & Vilardaga, 2015; Silva & Bilezikian, 2015).

Continuous infusions of PTH in rats have shown that PTH increases the mRNA encoding for RANKL and decreases the OPG mRNA. PTH also increases the expression of monocyte chemoattractant protein-1 (MCP-1), a chemokine for macrophages and monocytes. MCP-1 increases the chemoattraction of pre-osteoclasts and thus increases bone resorption (Silva & Bilezikian, 2015).

If PTH is administered in low, intermittent doses, its effects on the skeleton are anabolic, meaning it increases bone formation. For this reason, a synthetic analogue of the amino-terminal fragment of PTH is used in the treatment of osteoporosis

[teriparatide, rhPTH(1-34)]. When given intermittently, PTH acts on osteoblasts and promotes osteoblastogenesis, reduces their apoptosis and differentiates quiescence lining cells into active osteoblasts (Silva & Bilezikian, 2015).

The mediators that regulate the actions of PTH on bone formation are not fully understood. One of the possible mediators seems to be sclerostin, the product of the SOST gene, located on chromosome 17q12–q21 in humans. Sclerostin is a glycoprotein produced by osteocytes and it can inhibit the Wnt/β- catenin pathway and thus inhibit bone formation. This pathway is activated when a Wnt ligand binds to a dimeric receptor complex formed by the transmembrane Frizzled (Fz) receptor and its co-receptor, low-density lipoprotein receptor related protein 6 (LRP6) or its close relative LRP5. Sclerostin binds to the LRP receptors and inhibits the Wnt pathway and this leads to decreased bone formation. PTH, through the cAMP pathway, seems to inhibit the SOST gene, and thus reduces sclerostin. There is a negative correlation between circulating sclerostin and PTH levels and patients with primary hyperparathyroidism, a disease with an increase in PTH levels, have been found to have lower sclerostin levels. Results from studies evaluating whether dickkopf1 (Dkk1), another inhibitor of the Wnt pathway, is also a mediator of PTH action are controversial. Another potential mediator of the PTH action is EphrinB2 which stimulates the interaction between two osteoblastic cells (Silva & Bilezikian, 2015).

1.1.2.3.2 Effects on kidneys

PTH has effects on the kidneys at several levels; at the proximal tubule (65% reabsorption of calcium) it increases the 1α-hydroxylase activity, thus increases the production of the active form of vitamin D [1,25$(OH)_2$D]. At the same level, it inhibits

the apical type 3 Na^+/H^+ exchanger and the Na^+/K^+-ATPase located in the basolateral membrane, thus inhibiting the Na^+ and $HCO3^-$ reabsorption. It can also inhibit the sodium dependent phosphate cotransport by inhibiting NaPi-IIa and NaPi-IIc, leading to phosphaturia. Approximately 20% of the calcium is reabsorbed at the thick ascending limp of the loop of Henle. In this part of the kidney, the calcium sensing receptor described in more detail later, also has a role in the regulation of calcium, by antagonising the hypercalcaemic actions of PTH. Therefore, if there is a raised extracellular fluid (ECF) calcium in the nephron, this is sensed by the receptor which in turn antagonises the PTH action. Elevated ECF calcium decreases the activity of the Na/K/2Cl cotransporter and thus decreases the calcium paracellular reabsorption. That is why it is considered to act in an analogous way to loop diuretics. A further 15% of the calcium is absorbed from the distal tubule. At this level, PTH can actually influence calcium levels directly, by stimulating the calcium transfer in the renal tubule via the transient receptor potential cation channel (TRPV5), by assisting the transport of calcium through the cell with proteins like calbidin-D28K and by assisting the transfer of calcium into the blood through the Na^+/Ca^{2+} exchanger (NCX1) (Favus & Goltzman, 2013; Gardella & Vilardaga, 2015; Vautour & Goltzman, 2018).

1.1.2.3.3 Effects on intestine

PTH acts directly on the calcium levels, as described above, but also it has an indirect action though the increase of $1,25(OH)_2D$. The active form of vitamin D acts at the intestine where calcium is absorbed (90% in duodenum and jejunum). Calcium is absorbed both with an energy-dependent pathway (10-15% of the dietary load), mainly regulated by vitamin D and a passive paracellular path regulated by electrochemical

gradients. The energy-dependent pathway includes the transfer of calcium into the intestinal cell via the transient receptor potential cation channel (TRPV6), then the transport of calcium through the cell via the annexin2 calbidin D9K, and finally the extraction of calcium through the basolateral membrane into the blood via the PMCA1b. Vitamin D increases the expression of these proteins. Vitamin D can also regulate claudin 2 and claudin 12, both parts of paracellular channels, and enhance the passive absorption of calcium. However, this absorption is reduced in cases of high dietary intake (Favus & Goltzman, 2013; Vautour & Goltzman, 2018).

1.1.2.4 Metabolism

PTH is cleared by both the liver (40-75%) and the kidney (20-30%) and has a half-life is approximately 2-4 minutes (Gardella et al, 2010). Moreover, as mentioned above, PTH is degraded into different fragments, known as N and C-terminal fragments. This is important because, firstly, their existence can affect the measurement of the hormone (as described below) and, secondly, it has been suggested that these fragments might have a biological action. C-terminal fragments are secreted by the parathyroids, along with the intact hormone. The ratio of fragments to intact hormone is increased in the case of hypercalcaemia and reduced in the case of hypocalcaemia. It has also been suggested that the pattern of proteolysis depends on the levels of calcium. Although circulating PTH is cleared by both the kidney and the liver, the kidneys are not able to produce fragments of the hormone, just clear them. On the contrary, the liver produces these fragments in the Kupffer cells and they can re-enter the circulation (Murray et al, 2005).

The existence of N-fragments has also been studied. It has been suggested that are produced in the liver and rapidly degraded and do not normally exist in measurable quantities in the blood but can be found in renal failure, hyperparathyroidism or both (Murray et al, 2005).

1.1.3 Calcium-sensing receptor

The relationship between calcium and PTH is described by a sigmoidal curve (Figure 1-4).

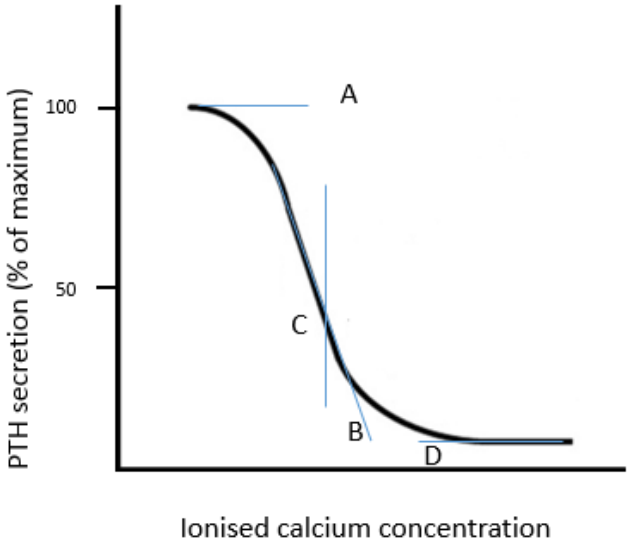


Figure 1-4: Sigmoidal curve characterising the inverse relationship between ionised calcium and PTH.

There are four characteristic points. A: maximum secretion rate of PTH in hypocalcaemia. B: slope at midpoint. C: level of calcium at the midpoint or the level of calcium needed to suppress 50% of PTH secretion. D: minimum secretion rat in hypercalcaemia

PTH is secreted by the parathyroid glands which act like a “calciostat” in the human body; its secretion is regulated by the calcium-sensing receptor (CaSR). The receptor

belongs in the G-protein coupled receptor (GPCR) superfamily and it is a member of the subgroup known as family C, characterised by the presence of a large extracellular domain. It was first cloned from bovine parathyroid glands in 1993 and in 1995 in humans. The receptor can be found mainly in the parathyroid cells, but also in other organs like the kidneys, gastrointestinal tract, bones, nervous system, breast, heart and in cells like the epidermal. The receptor senses the levels of circulating calcium by binding extracellular calcium in a "venus flytrap" way (Figure 1-5) and through complex intracellular signalling pathways, it adjusts the levels of PTH. It has three parts: a large 612-amino acid large extracellular one, a 250-amino acid transmembrane domain and a 216-amino acid C-terminus tail (Magno et al, 2011). The CaSR's gene is located on chromosome 3q21.1 (Hannan & Thakker, 2013).

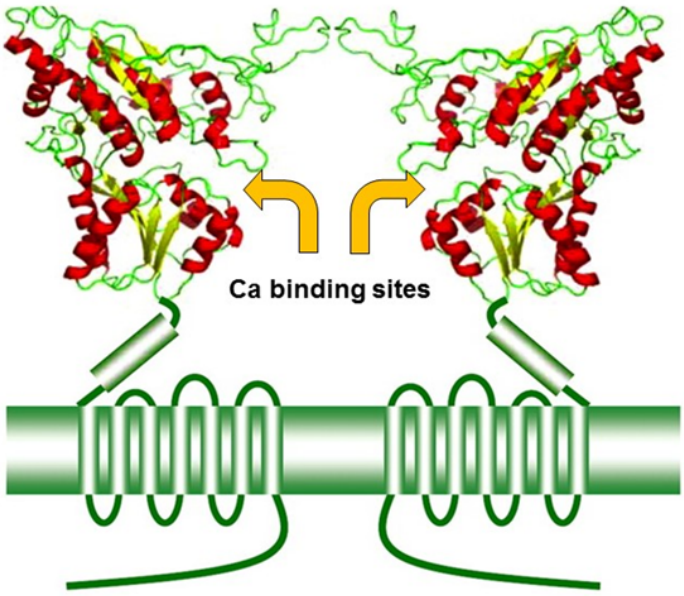


Figure 1-5: Schematic model of the extracellular part of the CaSR.

The model is based on the related structure of the metabotropic glutamate receptors (mGLUR1). The receptor is presented as a dimer; each monomer contains a "venus flytrap" part, used as a binding site for calcium. Slightly modified form from the original (Huang et al, 2007). Copyright: American Society for Biochemistry and Molecular Biology

Although calcium is the main agonist of this receptor, other substances have been found to stimulate it (Figure 1-6), either by direct by activation (type I) or by sensitizing the receptor to its agonists (allosteric modulators or type II). Pharmacological agents like cinacalcet, which is a calcimimetic, can enhance the sensitivity of the receptor to calcium, and are used to treat primary hyperparathyroidism (Magno et al, 2011).

The intracellular signaling includes several messengers. To start with, the receptor can act through phospholipases like the phospholipase C (PLC) pathway. It has also been suggested that it stimulates phospholipase D (PLD) and A_2 (PLA_2). The activation of PLA_2 and thus the release of arachidonic acid, is a result of different pathways. It can be activated both through PKC and by the release of intracellular calcium, as a result of the PLC pathway. Intracellular calcium stimulates calmodulin, which then stimulates calmodulin-dependent protein kinase (CaMK), both needed to activate PLA_2. Another pathway that is activated through the CaSR is the MAPK signaling pathway, which involves several proteins (tyrosine kinase Src, Ras, Raf, MEK, ERK). A further second messenger that is involved is cAMP. It has been shown that the receptor activation can inhibit cAMP, although there can be cell specific differences in the responses, thus some cells can actually increase the level of cAMP. Phosphorylation and activation of the protein kinase Akt which is antiapoptotic can also take place. Finally, the Rho pathway can be involved and that can ultimately activate phospholipase D. Recent studies have shown that there are several proteins that can interact with the receptor and either protect it from degradation (filamin A) or lead to its destruction (β-arrestin, dorfin) (Magno et al, 2011).

When CaSR is activated by increased calcium in the parathyroids, its role is to inhibit PTH secretion. The actual mechanism behind this is unknown. Possible mechanisms suggested include the rearrangement of the cytoskeleton to prevent the vesicles containing PTH to approach the membrane. CaSR also acts on the PTH mRNA and causes its destabilisation and may also have an post-translational effect on PTH by promoting the cleavage of the PTH molecule (Hendy, 2018).

On the other hand, CaSR is located mainly at the basolateral surface of the cells in the cortical thick ascending limb in the kidneys. In this part of the kidney, it antagonises the hypercalcaemic actions of PTH. Elevated ECF calcium decreases the activity of the Na/K/2Cl cotransporter and thus decreases the calcium paracellular reabsorption leading to hypercalciuria. In this way, hypercalcaemia causes excretion of sodium, chloride and water in the urine, thus causing dehydration. In the distal convoluted tubule, the receptor stimulates TRPV5, therefore it stimulates the reabsorption of calcium. In the cortical collecting duct, it enhances the secretion of H^+ to the urine, thus preventing nephrolithiasis. Finally, in the case of hypercalciuria, and in order to protect from nephrolithiasis, the CaSR located in the inner medullary collecting duct, inhibits the reabsorption of water mediated by vasopressin and thus dilutes the calcium in the urine. This is the reason why diabetes insipidus can be a result of hypercalcaemia (Hendy, 2018).

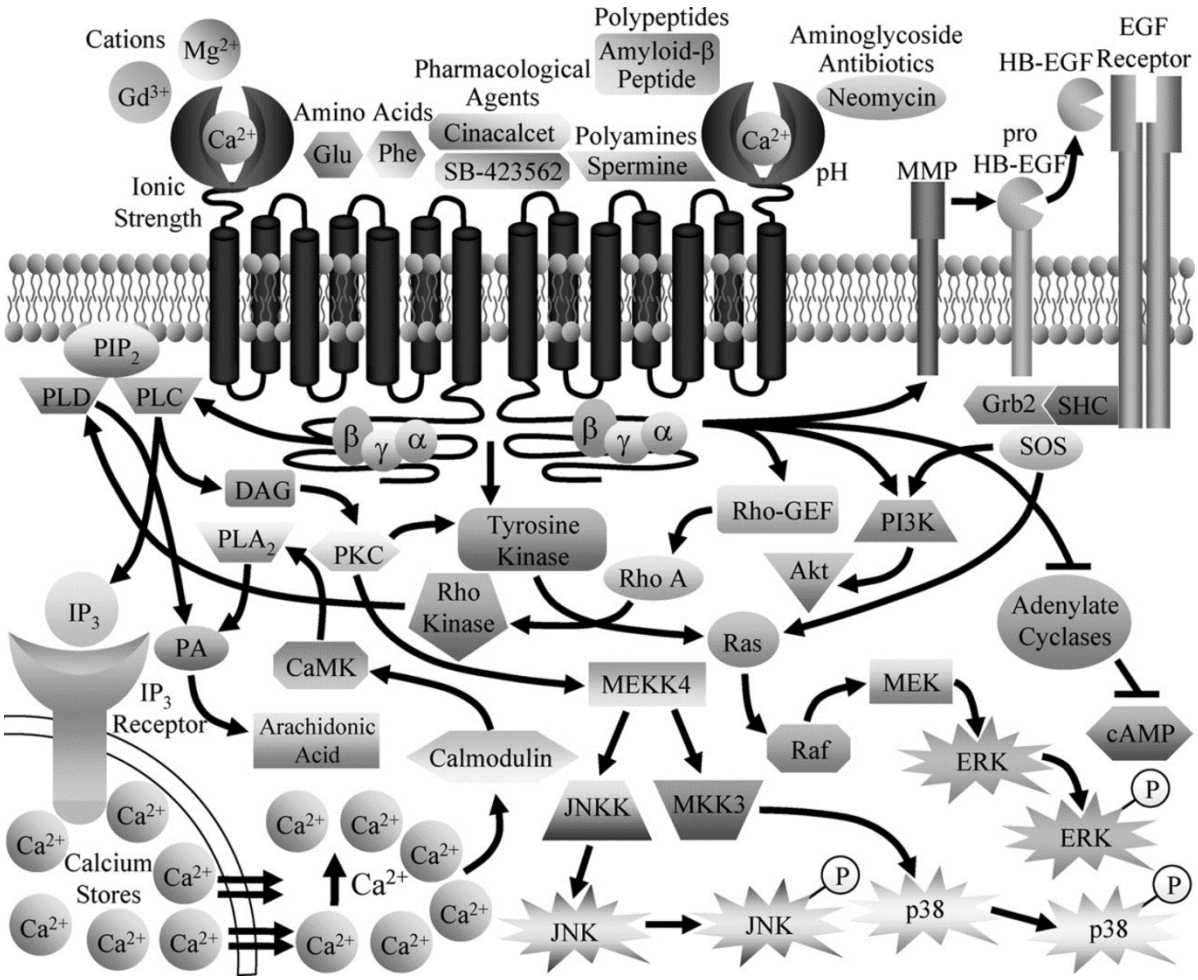


Figure 1-6: The intracellular signalling of the calcium sensing receptor.

DAG, Diacylglycerol; CaMK, calmodulin-dependent protein kinase; MMP, matrix metalloprotease; Rho-GEF, Rho- guanine nucleotide exchange factor. From: The Calcium-Sensing Receptor: A Molecular Perspective. Endocr Rev. 2011;32(1):3-30. doi:10.1210/er.2009-0043, Endocr Rev | Copyright © 2011 by The Endocrine Society

1.1.4 Vitamin D

Vitamin D is another important regulator of plasma calcium and the term refers to both ergocalciferol (vitamin D2) and colecalciferol (vitamin D3). The main source of vitamin D in the human body is the production of colecalciferol by the skin. The pro-vitamin 7-

dehydroxycholesterol (7-DHC), under the effect of ultraviolet B (UV-B) light, forms previtamin D3 and then isomerizes to vitamin D3. Another source of vitamin D is diet. D3 can be mainly found in fatty fish, whereas ergosterol (the pro-vitamin D ergocalciferol), can be found in mushrooms and can be irradiated into ergocalciferol by UV-B. Fortified foods also contain vitamin D. The dietary vitamin D is absorbed by chylomicrons in the small intestine. These forms of vitamin D however, are not biologically active (Holick et al, 2011).

Through a series of cytochrome P540 enzymes, vitamin D matures gradually into its active form. First, it is hydroxylased in the liver by 25-hydroxylase (CYP2R1 the most important) and forms 25(OH)D. This molecule is considered the best indicator for vitamin D status and has a half-life of 2-3 weeks. After formation, 25(OH)D is released into the circulation. A further 1α hydroxylation takes place in the kidneys by 1α-OHase (CYP21B1), to form the biologically active form, $1,25(OH)_2D$ or calcitriol (half-life 4 hours). As mentioned above, PTH stimulates this conversion (Holick et al, 2011). Both 25(OH)D and $1,25(OH)_2D$ are mainly bound to vitamin D binding protein (DBP). When released from this protein, $1,25(OH)_2D$ can act on its intracellular receptor (vitamin D receptor, VDR) (Pludowski et al, 2018).

Vitamin D regulates the calcium levels by its actions in the bones, intestine and kidneys. Osteoblasts have receptors for vitamin D and calcitriol can stimulate their differentiation to mature cells. It can also upregulate RANKL and downregulate OPG therefore, it can benefit bone resorption in an indirect way (Bikle et al, 2018).

The actions of vitamin D in increasing calcium through the intestine have been described above. Finally, vitamin D can enhance the PTH receptor expression in the kidneys. The effects of PTH and vitamin D in the kidney are synergistic. The levels of

active vitamin D are controlled by the kidneys, through a different hydroxylation than the one described above. The enzyme 24-hydroxylase (CYP24A1) is responsible for this process. Active calcitriol has a negative feedback loop of CYP21B1, while it stimulates CYP24A1. PTH on the other hand stimulates CYP21B1 (Bikle et al, 2018).

Finally, calcitriol has the ability to supress the activity of PTH and inhibits the proliferation of parathyroid cells. It can also upregulate the transcription of the CaSR at the parathyroids, thus sensitising the gland (Bikle et al, 2018).

Recently, there has been some controversy to what is defined as vitamin D sufficiency and deficiency and there have been various recommendations to what would be the ideal intake in the general population. The recommended intake of vitamin D was 200 IU/day up to 2010. This was because this dose would be enough to prevent rickets. In 2010, the US Institutes of Medicine (IOM) recommended 600 IU for adults <70 years of age and 800 IU for any adult above this age. They defined vitamin D deficiency as any level below 50 nmol/L (20 ng/ml) as this would be sufficient to prevent osteomalacia in at least 97.5% of the population (Ross et al, 2011). The Endocrine Society, defined deficiency as a level of 25(OH)D below 50nmol/L, while insufficiency was defined as a level of 50-75 nmol/L. They stated that adults might require at least 1500-2000 IU to raise vitamin D to the recommended limits (Holick et al, 2011).

There have been a variety of guidelines and recommendations since then, published in different countries and using different ways to what is considered normal. The IOM guidelines (issued in North America) were based on evidence regarding calcium and phosphate metabolism and bone health requirements. In contrary, the Endocrine Society, American Academy of Developmental Disability and the Central European recommendations were developed using the available the evidence on both skeletal

and the non-skeletal effects of vitamin D (Pludowski et al, 2018). Higher levels of vitamin D have been correlated with higher bone mineral density results, improvement in strength and reduction of falls and levels of at least 75 nmol/L have been reported to be related to antifracture efficacy (Holick et al, 2011).

Vitamin D deficiency is a cause of secondary hyperparathyroidism, i.e. elevated PTH. The levels of vitamin D needed to normalise the values of PTH, are considered an indicator of what can be classified as deficiency. There have been studies that showed that PTH levels have an inverse relationship with vitamin D and a plateau in PTH is starting to be observed in levels of 25(OH)D above 75 nmol/L but not all studies agree with this finding (Figure 1-7) (Holick et al, 2011).

Another approach to define the ideal vitamin D threshold is by evaluating the effect of supplementation with vitamin D on the levels of PTH. A threshold of 50 nmol/L has been suggested to be sufficient to lower PTH levels (Malabanan et al, 1998).

In the UK, both the National Osteoporosis Society and the National Institute for Health and Care Excellence (NICE) recommend a cut-off of 50nmol/L to define sufficiency (Aspray et al, 2014; NICE, 2018).

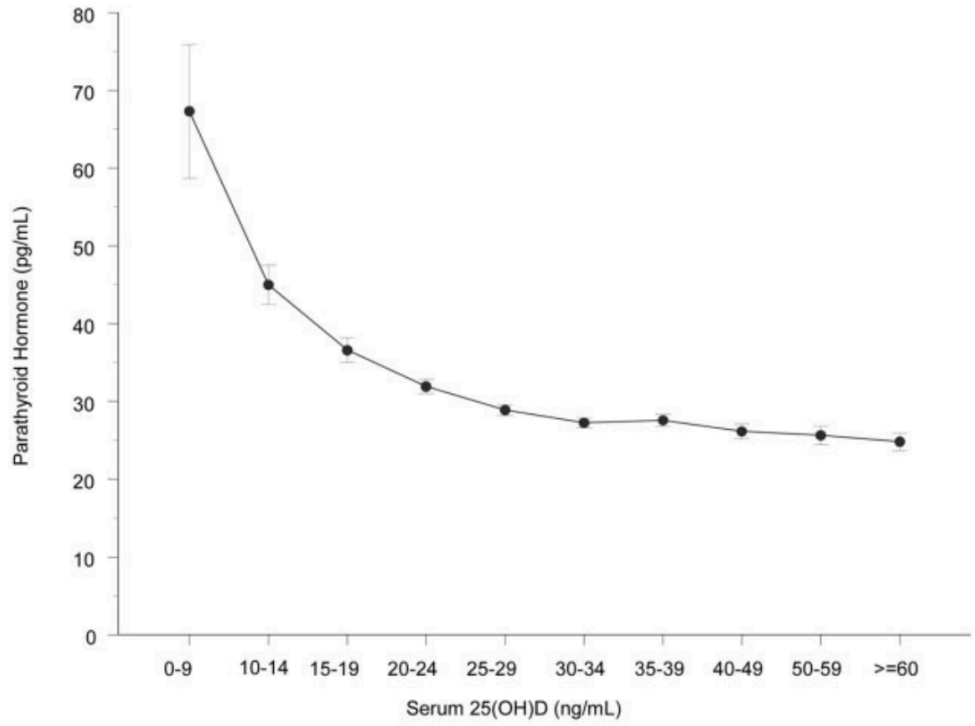


Figure 1-7: PTH concentration against 25(OH)D.

Serum PTH values began to increase with 25(OH)D concentrations less than 29.8 ng/ml. Figure from Holick et al, Prevalence of Vitamin D Inadequacy among Postmenopausal North American Women Receiving Osteoporosis Therapy, The Journal of Clinical Endocrinology & Metabolism, 2005, 90(6): 3215-3224 by permission of Oxford University Press (Holick et al, 2005)

1.1.5 Biochemical assessment of calcium homeostasis

1.1.5.1 Measurements

1.1.5.1.1 PTH Measurement

PTH measurement has evolved over the past years. The first-generation PTH radioimmunoassays (RIAs) were introduced in the 1960s and 1970s and they included multivalent antibodies against PTH of different species. These assays mainly reacted with the carboxyl-domain of PTH (amino acids 53-84) or the mid carboxyl-domain (amino acids 44-68) and thus identified inactive C-PTH fragments in a large extent

(approximately 80%). Since these fragments can be produced in chronic hypercalcaemia, and they are also accumulated in chronic kidney disease (CKD), it was difficult to distinguish non-PTH induced hypercalcaemia from mild PHPT and it was also difficult to interpret the results in chronic kidney disease. Comparing results from different assays was also a challenge. Second generation immunoradiometric assays (IRMA), also known as “intact PTH assays”, were introduced a few years later. The principal behind these assays was to use an antibody to capture the carboxyl-domain of human PTH (39-84) and detect the amino-domain (1-34) with a second labelled antibody. These methods allowed comparisons between studies, distinction between patients with chronic hypercalcaemia and mild PHPT and were easier to use in chronic kidney disease. However, it was subsequently proven, that these assays cross-react with large amino-truncated PTH fragments that are present in the circulation. The most abundant ones are the (7-84) and the (15-84). In normal subjects, these fragments represent 20% of the immunoreactivity, but in patients with chronic kidney disease, they represent up to 50%. Hence, in 1999, the third generation PTH assays were introduced, also known as the “whole PTH assays”. The capture antibody aimed against the carboxyl domain (39-84), but the labelled antibody in this case, aimed against a smaller region in the amino-terminal (1-4). However, it was shown that these assays also detect a modified PTH (1-84) molecule, formed post translationally, and characterised by phosphorylation of serine 17. This form represents <10% of the PTH values in normal subjects and approximately 15% in CKD patients. It can also be produced in higher concentrations in some patients with severe hyperparathyroidism and parathyroid carcinoma (Eastell et al, 2009). The diagnostic sensitivity for PHPT is similar between the second and third generation assays (Eastell et al, 2014).

The measurement of PTH can be performed using a random sample (NICE, 2019). The preferred way is by using an EDTA tube, as it is more stable in room temperatures (Roche, 2019). More details about PTH measurement can be found in Chapter 2.

1.1.5.1.1.1 Factors that affect the PTH level

The levels of PTH determine whether there is a parathyroid disorder or not, therefore, the use of the correct reference intervals of great importance. It has been recently suggested that reference intervals have to be established for second- and third-generation PTH assays using population studies with vitamin D-replete individuals. Moreover, the importance of stratifying these intervals according to age, sex, race, glomerular filtration rate, and body mass index (BMI) has also been discussed (Eastell et al, 2014).

In order to establish reference ranges for PTH a lot of things have to be taken into account. Any conditions that could have an effect on the level of PTH have to be excluded. As mentioned before, vitamin D deficiency is a cause of secondary hyperparathyroidism, i.e. elevated PTH. The levels of vitamin D needed to normalise the values of PTH, are considered an indicator of what can be classified as deficiency (Holick et al, 2011). When excluding patients with low vitamin D, the upper normal limit is found to be decreased (Souberbielle et al, 2017). However, as mentioned in the relevant vitamin D section, the cut-off for vitamin D deficiency is debated.

Decreased kidney function should also be an exclusion criterion, with eGFR<60 ml/min/1.73m^2 being the appropriate cut-off, as PTH tends to increase higher than this level. PTH also seems to increase in cases of chronic low calcium intake (Souberbielle et al, 2017).

Moreover, other factors seem to affect the PTH level. PTH is higher in obese people, and in black individuals when compared to white. However, in both cases, this effect could be due to lower 25(OH)D in all these individuals (Souberbielle et al, 2017).

Several studies have shown that PTH is increasing with age. Several factors have been speculated to explain this phenomenon. Some are decreasing renal function, reduced oestrogen, decrease in the number of vitamin D receptors in the intestine and thus reduced absorption of calcium, along with reduced responsiveness of 1α hydroxylase to PTH with age (Carrivick et al, 2015; Need et al, 2004).

Overall, age seems to be an independent predictor of PTH level. Need *et al* found that age, weight, ionised calcium and 25(OH)D, were all independent predictors, with 25(OH)D being the most significant (Need et al, 2004). A few years later, a group of researchers in Denmark found that vitamin D had the highest correlation coefficient, followed by BMI and age. Renal function did not reach statistical significance. In total, 16% of the PTH variability was explained by these factors. They also found that PTH levels started to increase at 25(OH)D <82 nmol/L (Rejnmark et al, 2011). A study performed using laboratory databases showed, that PTH increased with age and this increase was independent of 25(OH)D, ionised calcium, phosphate, and renal function. They found that each 10-year increase in age was associated with a 5% increase in PTH. In a subgroup analysis including only individuals with eGFR of ≥60 mL/ min/1.73m^2 and 25(OH)D of ≥50 nmol/L, they found that each 10-year increase in age was associated with a 6.1% increase in PTH (Carrivick et al, 2015). A recent study from France confirmed that PTH increases with age and this is independent from vitamin D status and kidney function. When studying only subjects with vitamin D ≥7

5nmol/L, they found that only age remained significantly associated with PTH (Souberbielle et al, 2016). Further details on these studies can be found in Table 1-1.

Publication	Recruitment	Factors included in linear regression	Independent predictors of PTH
(Need et al, 2004)	Evaluating 918 postmenopausal women attending an osteoporosis center. Excluded individuals on treatment for osteoporosis, or other medication or disease affecting calcium metabolism.	Age, weight, calcium absorption, ionised calcium, 25(OH)D, and creatinine	25(OH)D was the most significant followed by calcium, weight and age
(Rejnmark et al, 2011).	2316 women aged 17–84 years and measured intact PTH between 8:00 and 13:00. They excluded women on treatment with drugs known to affect calcium homoeostasis (thiazides, loop diuretics, lithium, glucocorticoids, anticonvulsants and anti-osteoporotic drugs). They also excluded participants with creatinine levels >150 μmol/L.	25(OH)D, age, BMI, calcium intake, total calcium, smoking	Positive correlation with age, BMI Inverse correlation with total daily calcium intake, plasma calcium and 25(OH)D levels. PTH levels were also lower in smokers Vitamin D had the highest correlation coefficient, followed by BMI and age
(Carrivick et al, 2015).	Laboratory databases in Western Australia (n= 17275 participants, mean age 60 years, 80% female). The exclusion criteria were history of osteoporosis, hypoparathyroidism or hyperparathyroidism, ionised calcium or phosphate outside the reference range, eGFR< 30 mL/min/1.73m^2 and age <20 years. As this was a study based on laboratory data, information on medical history was limited. Information on factors like ethnicity, BMI, menopause status, calcium intake, and use of medications was lacking	Sex, ionized calcium, 25(OH)D, phosphate, and eGFR	The increase in PTH with age is independent of 25(OH)D, ionised calcium, phosphate, and renal function

(Souberbielle et al, 2016).	This study evaluated 898 healthy subjects aged 18–89 years with a normal BMI (18-28.5 kg/m2) and eGFR. They excluded participants with a history of thyroid, renal, hepatic, cardiovascular, pulmonary, intestinal or psychiatric disorders, cancer, epilepsy, intercurrent illness occurring during the week preceding inclusion, current consumption of tobacco or other toxics, and treatment potentially modifying calcium/phosphorus metabolism.	25(OH)D, phosphate, calcium and eGFR, age and BMI	Vitamin D level and age When studying only subjects with vitamin D ≥75nmol/L, only age remained significantly associated

Table 1-1: Recent studies evaluating the effect of different factors on PTH levels

1.1.5.1.2 Total calcium measurement

Serum calcium is measured with colourimetric assays using different reagents to induce the change in colour. The colour intensity resulting from the chemical interaction is directly proportional to the calcium concentration and can be measured photometrically. The measurement of calcium is described further in the methods section.

Several causes of contamination can cause falsely elevated serum calcium levels and falsely low levels are seen more rarely. In the case of dehydration or haemoconcentration during venepuncture, where albumin increases, there are falsely elevations of the total calcium (Goldstein, 1990). This is a theoretical concern and it unlikely that this difference is clinically significant. Moreover, it is not practical to follow in everyday practice.

1.1.5.1.3 Albumin-adjusted calcium calculation

Total calcium can vary significantly depending on protein concentration as approximately forty percent is protein bound, mainly to albumin. Adjusting total calcium for albumin concentration is therefore a more practical means of determining serum calcium (Barth et al, 1996).

1.1.5.1.4 Ionised calcium measurement

Ionised calcium can be measured in plasma, serum or whole blood. Unfortunately, there are not any commercially available auto analysers, and the measurement is usually performed in blood gas analysers. Measuring ionised calcium can be a

challenge. This is due mainly to influences from the pH. Even the slightest exposure to air and loss of CO2 by non-anaerobic handling, can increase the pH (alkalosis) and thus decrease the ionised calcium. Moreover, if the analysis is not rapid, the production of lactic acid by the red blood cell glycolysis would decrease the pH and thus increase the ionised calcium. There are also limitations due to binding of calcium to the common anticoagulants. As mentioned above, the measurement is usually performed in gas analysers and this is problematic because of frequent electrode dysfunction. The performance depends on staff training and quality control standards. The instrument must be perfectly calibrated, and the measurement has to be performed immediately after the collection. Finally, the lack of well-established reference ranges, does limit its use (Baird, 2011; Boink et al, 1991; Brandi et al, 2016; Crowley & Gittoes, 2016).

1.1.5.2 Variability of measurements

When a test is repeated more than once, the results will rarely be similar. There are three sources of variation: pre-analytical, analytical and within subject biological variation. Pre-analytical variation relates to patient preparation and can be affected by factors like fasting, exercise and posture. It also relates to the different processes of collection and handling like, amongst others, whether an anticoagulant was used, whether the sample was stored or not and whether a tourniquet was applied (Fraser, 2013).

The analytical source of variation is related to the technique used and can be subdivided into random variation or precision (repeated analysis of the same sample) and systematic variation or bias. Random variation can be a result of the variability in

the sample volume as a result of pipetting, changing of temperature and environmental conditions. The random measurement variation follows a Gaussian distribution by definition. Thus, the coefficient of variation (CV) can be calculated. This is the ratio of the SD by the mean and multiplied by 100. If a method has a good precision, this means that the random variation is low. On the other hand, bias is the difference between the results obtained and an estimate of the true value. These values are usually given in proficiency testing programs (PT) or external quality assessment schemes (EQAS) (Fraser, 2013).

The third source of variation is the within patient biological variation. There are changes to many analytes throughout life for example gonadal hormones. Another source of variation is the circadian rhythm, where the analyte differs according to the time of the day. Some analytes also have monthly and seasonal cycles (Fraser, 2013).

The biological variation can also just be described as the random fluctuation around a homeostatic setting point. This is called the within-subject or intra-individual biological variation. When the same analyte is measured in different people, each one would have a different setting point. This is represented by the between-subject or inter-individual biological variation (Fraser, 2013).

1.1.5.2.1 Variability of PTH

There seems to be a circadian rhythm of PTH that has two peaks and two troughs (Figure 1-8). There is a trough mid-morning (10-11am), a peak late afternoon (5-6pm), a trough evening (8-9pm) and a peak early morning (2-3 am) (Calvo et al, 1991; el-Hajj Fuleihan et al, 1997). The underlying rhythm is mainly endogenous and the

amplitude of change is reported to be between 4- 10 pg/ml (el-Hajj Fuleihan et al, 1997).

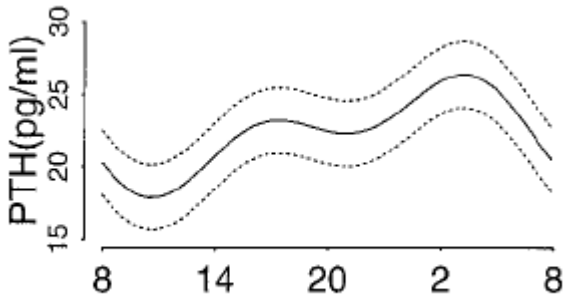


Figure 1-8: The daily variation of PTH.

Figure el-Hajj Fuleihan G, Klerman EB, Brown EN, Choe Y, Brown EM, Czeisler CA. The parathyroid hormone circadian rhythm is truly endogenous--a general clinical research center study. J Clin Endocrinol Metab 1997; 82:281-286, by permission of Oxford University Press

The circadian rhythm has been found to have alterations in women with osteoporosis (no nocturnal increase of PTH), female gender (late evening increase in PTH is blunted), prolonged fasting (no nocturnal rise after a 96-hour fast) and it has also been found to be lost in patients with PHPT (Calvo et al, 1991; Fraser et al, 1998)

As far as the seasonal variability of PTH is concerned, this is affected by the levels of vitamin D and this association is inverse. 25(OH)D is lower during winter and early spring months and higher in summer and early autumn. PTH on the other hand, is lower in the summer and higher in the winter (Rapuri et al, 2002).

1.1.5.2.2 Variability of calcium

There is a circadian rhythm for ionised calcium, with a peak in the morning. This rhythm differs by gender. A study in young men (17-30 years) several years ago, showed that there is a peak in ionised calcium late morning (~10am) and a nadir in the afternoon (~16:30) (Markowitz et al, 1981; Markowitz et al, 1988) (Figure 1-9).

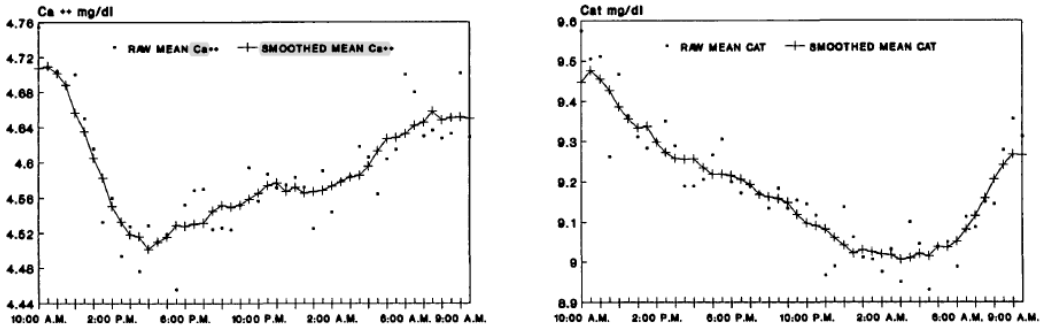


Figure 1-9: Variations of ionised (left) and total calcium (right) in health men.

Figure from Markowitz ME, Arnaud S, Rosen JF, Thorpy M, Laximinarayan S. Temporal interrelationships between the circadian rhythms of serum parathyroid hormone and calcium concentrations. J Clin Endocrinol Metab 1988; 67:1068-1073, by permission of Oxford University Press

Another study a few years later, in 25 women and 24 men (20-69 years), showed that there is a circadian rhythm in both sexes, but the pattern was different especially early morning (6-8am), with iCa decreasing in women and reaching a plateau in men. The mean maximal change was 0.066 mmol/L for women and 0.058 mmol/L for men (Figure 1-10). In women, the mean serum ionised calcium correlated significantly with total daily calcium intake (women more dependent on calcium intake than men) (Calvo et al, 1991).

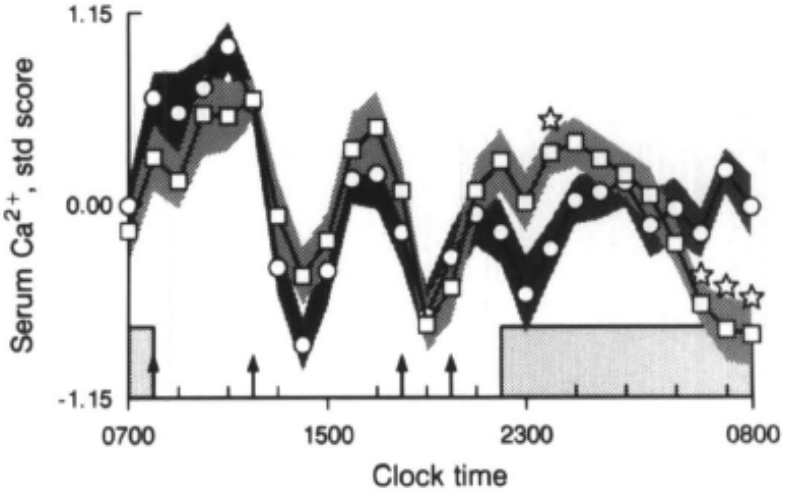


Figure 1-10: Circadian variation in serum iCa.

Men are shown in black, while women in grey. Meals were taken at times indicated by arrows, and a snack was consumed at 2000 h in some, but not all. The stippled horizontal bar indicates that the subjects were recumbent. Figure by Calvo MS, Eastell R, Offord KP, Bergstralh EJ, Burritt MF. Circadian variation in ionized calcium and intact parathyroid hormone: evidence for sex differences in calcium homeostasis. J Clin Endocrinol Metab 1991; 72:69-76, by permission of Oxford University Press

Ionised calcium is also affected by food ingestion but remains stable overnight (Eastell et al, 1992).

On the contrary, total calcium seems to be affected by postural changes, with a peak once again late morning and a nadir starting in the evening and persisting until early morning (1-6am). (Markowitz et al, 1981; Markowitz et al, 1988) (Figure 1-9). This early morning decrease corresponds closely to a reduction in protein. The average increase in plasma volume during an hour's rest in the horizontal position was found to be 9% in healthy ambulatory subjects (Pedersen, 1972). A change from supine to a standing position can result in an increase of 0.05 to 0.20 mmol/L. Food intake can also affect the result slightly, with an increase as much as 0.15 mmol/L. Men in general

have slightly higher calcium levels than women (0.02 to 0.05 mmol/L) (Goldstein, 1990).

Section 2: Parathyroid gland disorders

Primary hyperparathyroidism (PHPT), is a classic endocrine calcium abnormality, and the most common cause of hypercalcaemia; along with malignancy-related hypercalcaemia, they constitute 90% of all the presentations of hypercalcaemia (Walsh et al, 2016). PHPT is characterised by parathyroid gland hyperfunction expressed with hypercalcaemia and increased levels of PTH.

This disease was first characterised in the beginning of the 20th century and since then its presentation has evolved significantly, from a symptomatic disease, to an asymptomatic form and finally, to what is now called normocalcaemic hyperparathyroidism (NPHPT).

1.2.1 Classical primary hyperparathyroidism: the first era. A historical overview

Diseases of the bones were described before their connection to the parathyroid glands. The German pathologist Friedrich Daniel von Recklinghausen described the bone disease called “osteitis fibrosa cystica” in 1891. Max Askanazy described a patient with this disease and the existence of a parathyroid tumour in 1903 but the first who suggested their connection, was Jacob Erdheim in 1907. He claimed that the enlargement of the parathyroid glands is a compensatory process to the skeletal disease. It was not until 1915 when Friedrich Schlagenhaufer observed that one gland was enlarged in this disease, thus proposed that this was the primary event causing the bone disease. Having that in mind, the suggested treatment would be parathyroidectomy. The first parathyroidectomy to treat a bone disease, was performed ten years later (1925) in Vienna, by Felix Mandl. His patient was a 38-year old man, called Albert Jahne who at the age of 34 developed leg pain and fatigue. At

36 he had osteopenia and cysts in his X-rays. He developed a femur fracture about a year later. Mandl found high serum and urine calcium and tried to treat him with parathyroid extract and parathyroid tissue transplant. This worsened the patient's symptoms, so Mandl decided to operate. A parathyroid tumour was removed and the operation resulted in the reversal of the bone changes. The patient had a recurrence seven years later and died of renal failure. Within the next few years, several surgeons treated their hyperparathyroid patients with surgery. The most famous case of PHPT was that of captain Martell (Figure 1-11). His disease was presented by Fuller Albright, a person that devoted his life in the study of parathyroid diseases. The patient was operated twice in 1926 without success. A few years later (1932), Edward Churchill and Oliver Cope opened the chest of the captain after his request and managed to find a gland there. The captain died of renal failure a few weeks after the operation (Dorairajan & Pradeep, 2014; Eknoyan, 1995; Kafetzis et al, 2011).

Figure 1-11: Captain Martell, the most famous patient with PHPT

Classical hyperparathyroidism, described as a disorder affecting "stones and bones" and having "abdominal groans and psychic moans", included symptomatic

hypercalcaemia (nausea, vomiting, polyuria, constipation, anorexia, electrocardiogram findings like short QT, coma) and involvement of the skeleton (osteitis fibrosa cystica) and the kidney (recurrent kidney stones and nephrocalcinosis). Symptoms and signs from other organs and systems could also be present: cardiovascular (arrhythmias, left ventricular hypertrophy, hypertension, atherosclerosis and valve calcification), gastrointestinal (peptic ulcer, pancreatitis), muscular (muscle weakness) and psychiatric (anxiety, psychosis, apathy, irritability) (Bandeira & Bilezikian, 2016). The classic radiological findings included salt and pepper degranulation of the skull, subperiosteal bone resorption, distal clavicle tapering, bone cysts and brown tumours (Silva et al, 2018).

1.2.2 Asymptomatic primary hyperparathyroidism: the second era

During the last decades, asymptomatic PHPT has been the main clinical phenotype, mainly because of the more frequent laboratory investigations and hence, earlier diagnosis. The reported prevalence of PHPT varies in the literature and is reported to be 0.89% in the United States and 1.07% in Europe. It affects women more than men (approximately 4:1 ratio) and the disease usually presents after menopause (Khan et al, 2017; Silva et al, 2018).

1.2.2.1 Aetiology

Single parathyroid adenoma is identified in about 80-85% of patients. Four gland hyperplasia is diagnosed in about 15-20% of PHPT patients and can occur sporadically or in the clinical set of genetic syndromes. Parathyroid carcinoma is rare (<1%) (Silverberg, 2013). Factors associated with developing PHPT include low

calcium intake, low physical activity, higher body weight, hypertension, furosemide use, external radiation to the neck and use of lithium (Silva et al, 2018).

In most patients, PHPT presents as a sporadic disease (in 90-95% of cases). Although hereditary forms are not so common, they are important to consider when PHPT is diagnosed in young ages, when there is a family history of hypercalcaemia, when there are other tumours associated with syndromes or when there is multiglandular disease (Bandeira & Bilezikian, 2016; Khan et al, 2017). These forms can be seen in Table 1-2.

Disorder	Gene/ protein	Clinical presentation
Multiple Endocrine neoplasia (MEN) type 1	MEN1 (menin) Autosomal dominant	PHPT 95% Endocrine pancreatic tumours Pituitary adenomas Other: adrenocorticoid or carcinoid tumours, lipomas, collagenomas, cutaneous angiofibromas
MEN2	RET Autosomal dominant	PHPT 20% Medullary thyroid carcinomas Pheochromocytomas
MEN4	CDKN1B Autosomal dominant	PHPT 80% Pituitary tumours Endocrine pancreatic tumours Other: adrenal, gonadal, thyroid and renal tumours
HPT-JT (hyperparathyroid jaw-tumour syndrome)	CDC73 (parafibromin) Autosomal dominant	PHPT but high prevalence of carcinoma (15%) Fibromas of mandible and maxilla Renal and uterine tumours

FIHP (familial isolated hyperparathyroidism)	MEN1 (menin), CDC73 (parafibromin), CASR, GCM2, CDKN1B (p21), CDKN2B (p15), CDKN2C (p18) Autosomal dominant	Isolated PHPT
NSHPT (neonatal severe primary hyperparathyroidism)	CASR Autosomal dominant or recessive	Neonatal PHPT
Non-syndromic PHPT (nsPHPT)	PTH	

Table 1-2: Hereditary forms of primary hyperparathyroidism.

Adapted from original (Eastell et al, 2014; Silva et al, 2018). CDC73: cell division cycle 73; CDKN: cyclin-dependent kinase inhibitor; RET: rearranged during transfection proto-oncogene

1.2.2.2 Clinical presentation

Primary hyperparathyroidism mainly affects the kidneys and the skeleton. The most common renal feature observed now in patients with the asymptomatic form is nephrolithiasis, present in about 7-20% approximately. Other consequences of renal involvement are nephrocalcinosis and reduced kidney function (Khan et al, 2017).

An accelerated bone loss can also be observed, especially at the distal 1/3 radius which is mainly formed by cortical bone. This can be seen in everyday practice by measuring the bone mineral density (BMD). The spine, which is mainly a trabecular site, is more preserved (Silverberg et al, 2014). PHPT patients appear to have an increased risk of fractures, especially vertebral (Khan et al, 2017)

These patients could also present with neurocognitive features, like anxiety, poor concentration and reduced quality of life. Moreover, even with the asymptomatic form,

endothelial dysfunction, left ventricular hypertrophy and vascular calcification can be present resulting in increased risk of cardiovascular mortality (Silva et al, 2018).

1.2.2.3 Diagnosis

As mentioned above, PHPT is presented with elevated levels of calcium and PTH. Another laboratory abnormality that can be observed is low-normal serum phosphorus; low levels can be observed in approximately one third of patients. Alkaline phosphatase (ALP), a bone formation marker, can be elevated or at the upper range of normal. Hypercalciuria can also be present in one third of patients. Elevated $1,25(OH)_2D$ levels can also be seen in 25% of patients, and they are usually accompanied by low normal 25(OH)D levels (Silverberg, 2013). The assessment of PHPT should include laboratory measurements [calcium, PTH, phosphate, ALP, renal function, 25(OH)D, 24-hour urine collection for calcium and creatinine], BMD measurement including the forearm and vertebral spine assessment. In case of high urine calcium, a stone profile should also be assessed and abdominal imaging to assess for renal stones should be performed (NICE, 2019; Silva et al, 2018).

1.2.2.4 Management

The management of asymptomatic PHPT has been revised over the last years and the patients need to meet any of the following criteria described in Table 1-3 to be advised for surgery. Surgery is also indicated in patients for whom medical surveillance is neither desired nor possible. The patient's choice for surgery is also an indication, as long as there are no medical contraindications (Bilezikian et al, 2014).

Surgery can have really high cure rates (95%) when performed by an experienced surgeon (Khan et al, 2017).

	1990	**2002**	**2008**	**2013**
Serum calcium (>upper limit of normal)	1–1.6 mg/dL (0.25–0.4 mmol/L)	1.0 mg/dL (0.25 mmol/L)	1.0 mg/dL (0.25 mmol/L)	1.0 mg/dL (0.25 mmol/L)
Skeletal	BMD by DXA: Z-score <−2.0 (site unspecified)	BMD by DXA: T-score <−2.5 at any site	BMD by DXA: T-score <−2.5 at any site	A. BMD by DXA: T-score < −2.5 at lumbar spine, total hip, femoral neck, or distal 1/3 radius
			Previous fragility fracture	B. Vertebral fracture by x-ray, CT, MRI, or VFA
Renal	A. eGFR reduced by >30% from expected	A. eGFR reduced by >30% from expected	A. eGFR < 60 ml/min	A. Creatinine clearance <60 ml/min
	B. 24-h urine for calcium >400 mg/d (>10 mmol/d)	B. 24-h urine for calcium >400 mg/d (>10 mmol/d)	B. 24-h urine for calcium not recommended	B. 24-h urine for calcium >400 mg/d (>10 mmol/d) and increased stone risk by biochemical stone risk analysis
				C. Presence of nephrolithiasis or nephrocalcinosis by x-ray, ultrasound, or CT
Age, years	<50	<50	<50	<50

Table 1-3: Guidelines for surgery in asymptomatic PHPT: a comparison of current recommendations with previous ones.

(Adapted from Bilezikian et al, Guidelines for the Management of Asymptomatic Primary Hyperparathyroidism: Summary Statement from the Fourth International Workshop, The Journal of Clinical Endocrinology & Metabolism. By permission of Oxford University Press)

Preoperative localisation is used to identify patients for minimally invasive parathyroidectomy (MIP) and when recurrence or persistence is suspected after surgery. The main imaging techniques used are either technetium-99m-sestamibi or parathyroid gland ultrasound. Computerised tomography (CT) and magnetic resonance imaging (MRI) are mainly used to detect ectopic parathyroid tissue (Silverberg, 2013).

The medical management of PHPT includes bisphosphonates, mainly alendronate, and the calcimimetic drug cinacalcet. Bisphosphonates aim at improving the BMD without affecting calcium levels. Cinacalcet on the other hand, does not affect the BMD, but can lower the calcium back to normal in many patients, and can result in a modest reduction of PTH (Bilezikian et al, 2014). The National Institute for Health and Care Excellence (NICE) suggests cinacalcet for patients where surgery is not an option and when their calcium is above 2.85 mmol/L with symptoms of hypercalcaemia or above 3.0 mmol/L regardless of symptoms (NICE, 2019). Nutritional advice should also be given in these patients, including adequacy of calcium intake and vitamin D supplementation in patients who are deficient (Silva et al, 2018).

1.2.2.5 Monitoring

Monitoring of this disease in patients who do not have surgery, also changed over the years. The different recommendations are summarised in Table 1-4. If the individual develops any indications for surgery during monitoring, then parathyroidectomy should be the next step (Bilezikian et al, 2014).

Measurement	**1990**	**2002**	**2008**	**2013**
Serum calcium	Biannually	Biannually	Annually	Annually
Skeletal	DXA, annually (forearm)	DXA, annually (3 sites)	DXA, every 1–2y (3 sites)	Every 1–2 y (3 sites) X-ray or VFA of spine if clinically indicated
Renal	eGFR, annually; serum creatinine, annually	eGFR, not recommended; serum creatinine, annually	eGFR, not recommended; serum creatinine, annually	eGFR, annually; serum creatinine, annually If renal stones suspected, 24-h biochemical stone profile, renal imaging by x-ray, ultrasound, or CT

Table 1-4: Guidelines for monitoring patients with asymptomatic PHPT who do not undergo parathyroid surgery: a comparison of current recommendations with previous ones.

(Adapted from Bilezikian et al, Guidelines for the Management of Asymptomatic Primary Hyperparathyroidism: Summary Statement from the Fourth International Workshop, The Journal of Clinical Endocrinology & Metabolism. By permission of Oxford University Press)

1.2.2.6 Natural history

In patients who do not have surgery, biochemical abnormalities can remain stable over the first 15 years, with calcium gradually starting to increase after the thirteenth year. BMD also remains stable for a few years and begins to decrease after the first eight years (Rubin et al, 2008).

Surgery results in the correction of biochemical measurements, improvements in BMD and lower rates of vertebral fractures. The risk of renal stones also decreases, but remains higher than that of the general population. Moreover, surgery prevents further decreases in the kidney function. Some studies have shown that cognitive symptoms could have an improvement (Khan et al, 2017).

1.2.2.7 Differential diagnosis

There is another disorder of calcium metabolism also causing high levels of PTH, called familial hypocalciuric hypercalcaemia (FHH) and it is crucial to distinguish PHPT from it, because the latter can be treated with surgery, while patients with FHH do not require parathyroidectomy. FHH is an autosomal dominant disorder, characterised by lifelong, non-worsening hypercalcaemia, accompanied by hypocalciuria and, in 10-15% patients, high PTH values. There are three forms of this disorder, FHH type 1, 2 and 3. FHH 1 (approximately 65% of the patients with FHH), is caused by loss-of-function mutations of CaSR; as a result, higher concentrations of calcium are needed in order to suppress the PTH release (Hannan & Thakker, 2013). FHH2 is caused by loss-of-function of the G-protein subunit a11 (encoded by the GNA11 gene, located on chromosome 19p13.3), which takes part in the CaSR signalling (Nesbit et al, 2013a). Finally, FHH3 is caused by adaptor protein-2 sigma subunit (AP2σ2)

mutations (gene AP2S1, chromosome 19q13.3), a component involved in the endocytosis of G-protein coupled receptors, and thus affecting CaSR levels on the cell surface (Nesbit et al, 2013b).

In everyday practice, PHPT and FHH are distinguished using the 24-h urine calcium to creatinine clearance ratio (CCCR):

CCCR= [24h urine Ca x serum creatinine] ÷ [serum Ca x 24h urine creatinine]

A cut off of <0.01 is used to identify FHH patients, while a value >0.02 is suggestive of PHPT. This technique has some limitations, since according to a study a few years ago, the cut point of <0.01 only identifies 65% of patients with FHH, and misclassifies 4% of patients with PHPT. Not all patients with PHPT have hypercalciuria with a CCCR of >0.02; that is because they either have a mild disease or vitamin D deficiency. This paper suggests a two-step procedure, with a cut off value of 0.02; this would identify 98% of the FHH patients and misclassify 35% of PHPT. The second step should include genetic studies (Christensen et al, 2008).

More recently, a new tool was developed, called Pro-FHH. The authors suggested the use of this tool to predict whether a patient has PHPT. In order to develop this tool, they used participants from two prospective cohorts, one in Paris, France and one in Aarhus, Denmark. Using these patients, they developed the following equation:

$$p = \frac{1}{1+e^{-23.19+11.17\times P_{Ca}-7.77\times \text{PTH ratio}-3.09\times \text{Ln}(24\text{h}-\text{CCCR})-2.89\times \text{Ln}(\text{MBR ratio})}}$$

MBR: Marker of bone remodelling (osteocalcin or ALP). For PTH and MBR, they used the ratio of measured value to upper limit of normal due to various analytical methods used throughout the study.

The area under the curve (AUC) for Pro-FHH was significantly higher (0.961) than that of 24h-CCCR (0.862) and using pro-FHH would had prevented unnecessary surgery in 2 patients out of 100 with FHH and unnecessary genetic testing in 21 out of 140 patients with PHPT. Although this method looks really promising to be used in everyday practice, its disadvantage is that it has only been tested in a population with normal PTH, because only 20% of FHH patients have high PTH (Bertocchio et al, 2018).

1.2.3 Normocalcaemic hyperparathyroidism: the third era?

As mentioned above, a specific phenotype of asymptomatic PHPT, called normocalcaemic hyperparathyroidism (NPHPT) was recognised in one of the last workshops on asymptomatic PHPT (Silverberg et al, 2009). It had been described by several researchers since the 1960s and was characterised by normocalcaemia with elevated PTH levels as described below.

In everyday practice, NPHPT is usually diagnosed during the evaluation of secondary osteoporosis and thus, mainly found in referral centres. Until now, there are limited data on the prevalence, causes, clinical presentation, natural history and consequences of this disease.

1.2.3.1 Definition

NPHPT is characterised by persistent normal calcium levels, accompanied by elevated levels of PTH on at least two consecutive measurements over a three to six-month-period (Eastell et al, 2014). It is important to have both the total and ionised calcium within the normal range, since cases of PHPT with normal total, but increased ionised calcium levels, have been reported (Wade et al, 2012). Other factors that cause high levels of PTH have to be excluded. These are:

- medications known to affect PTH levels (diuretics, lithium, denosumab, bisphosphonates, anticonvulsants)
- vitamin D insufficiency with 25(OH)D<50nmol/L (although levels >75nmol/L would be more desirable for the diagnosis)
- chronic kidney disease (eGFR <60 ml/min/1.73m^2)

- diseases of the gastrointestinal tract known to affect calcium absorption (coeliac disease, inflammatory bowel disease, bariatric surgery)
- renal calcium loss (hypercalciuria), defined by calcium excretion greater than 300mg/day in men, >250mg/day in women, or >4mg/kg body weight in either gender

(Eastell et al, 2009; Eastell et al, 2014; Yacobi-Bach et al, 2015)

As mentioned above, medications known to affect PTH levels have to be excluded when investigating NPHPT. During the Third International Workshop on Asymptomatic Primary Hyperparathyroidism, loop acting diuretics (furosemide) were included as a cause of secondary elevation of PTH (Eastell et al, 2009). On the contrary, the definition at the Fourth Workshop, included thiazides and not furosemide (Eastell et al, 2014). Sometime later, there was another publication in the same journal, commenting the definition used at this workshop and reviewing the literature on the effects of both thiazides and loop acting diuretics on PTH in humans; the authors suggested the removal of thiazides from the list of medications causing secondary hyperparathyroidism, since their effect on PTH has not been proven in humans (Yacobi-Bach et al, 2015).

Loop diuretics have been proven to increase PTH. In a study with postmenopausal women treated for at least two years with loop diuretics, treatment increased urinary calcium by 17% and PTH by 28% when compared to controls (Rejnmark et al, 2005). This result was consistent in a previous trial with a shorter duration of treatment (seven days). In the same trial, women treated with thiazides showed a non-significant increase of PTH and a significant increase of calcium reabsorption (Rejnmark et al,

2001). In another randomised, double-blinded trial of thiazides in subjects aged 60–79 years, no changes were found in the PTH levels (Ott et al, 2008).

Another topic of controversy, is the cut-off for vitamin D. In the UK, both the National Osteoporosis Society and the National Institute for Health and Care Excellence (NICE) recommend a cut-off of 50nmol/L to define sufficiency (Aspray et al, 2014; NICE, 2018).

1.2.3.2 Initial assessment for NPHPT

When a patient with normal calcium and high PTH is identified, the albumin-adjusted calcium should be calculated, ideally with an equation based on the local patient population. Ionised calcium would be appropriate to measure in the setting of really low albumin, where adjusted calcium could be unreliable. Vitamin D status should be assessed by using 25(OH)D and supplemented if low. Kidney function should also be assessed to exclude patients with CKD. A detailed history and medical examination should also be performed to assess poor calcium intake, causes of malabsorption and medication use (as defined above). Only if the above named causes of secondary hyperparathyroidism are excluded, can the diagnosis of NPHPT be made (Crowley & Gittoes, 2016)

1.2.3.3 Pathophysiology

1.2.3.3.1 Theory: NPHPT is the first phase of a biphasic disease course

There have been limited theories on the pathophysiology of this disease. It has been proposed that it is the first phase of a biphasic disease course, which can be followed

by hypercalcaemic hyperparathyroidism (Figure 1-12). The study suggesting this theory, recruited 22 patients from a referral centre with normal adjusted calcium and high PTH, confirmed on at least two occasions. Ionised calcium was measured in eight patients and it was normal. All the patients had 25(OH)D>20 ng/ml and the exclusion criteria were FHH, liver disease, renal disease, urinary calcium> 8.75 mmol/24h, gastrointestinal disease with malabsorption, metabolic bone disease, medications (lithium, thiazide, oestrogens, loop diuretics, bisphosphonates, anticonvulsants). The patients were referred due to osteoporosis (n=10), vertebral fracture (n=1) and kidney stone (n=3). The rest were self-referred. Four patients had mild hypercalciuria, seven had elevated levels of 1,25 dihydroxyvitamin D and phosphate was low in one patient. The researchers reported a positive relation between serum calcium and PTH (i.e. as calcium goes up, PTH goes up). This is a difference from the general population, where this relationship is inverse. The researchers conclude that NPHPT patients have abnormal PTH secretory dynamics (Silverberg & Bilezikian, 2003).

These patients were then followed for up to twelve months and three of them developed hypercalcaemia. These patients had higher PTH levels. One of them was operated and had two adenomas removed. After this finding of subsequent hypercalcaemia, they formed their assumption about the biphasic course (Silverberg & Bilezikian, 2003).

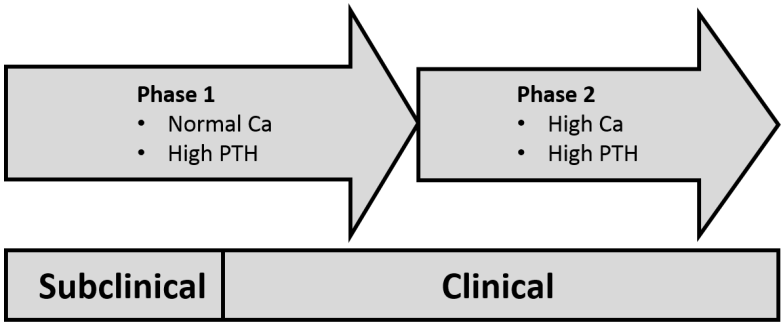


Figure 1-12: Proposed model for a biphasic disease course of primary hyperparathyroidism

Adapted form of the original (Silverberg & Bilezikian, 2003)

1.2.3.3.2 Theory: NPHPT is caused by a lower hypersecretion of PTH than in PHPT

This theory has been inconsistent between the different studies, with some supporting this finding (Castrillon et al, 2015; Diaz-Soto et al, 2015; Marques et al, 2011; Maruani et al, 2003; Siprova et al, 2016) and some contradicting it (Amaral et al, 2012; Koumakis et al, 2013; Tuna et al, 2015). More details for these studies can be seen in the table on the skeletal findings of NPHPT in the Appendix.

1.2.3.3.3 Theory: NPHPT is caused by PTH resistance

In a publication by Maruani and colleagues, it is proposed that NPHPT is caused by both kidney and bone resistance to PTH (target organs), as approximately 20% of the patients with elevated PTH, maintained a normal calcium level despite the similar level of PTH (Maruani et al, 2003).

The researchers retrospectively identified patients being diagnosed as primary hyperparathyroidism in their department (Department of Physiology in Paris) from 1990 to 1998. They identified 649 patients and then excluded 471 of them because of: 25(OH)D <15 nmol/L, magnesium deficiency (<0.71 mmol/L), impaired renal function (defined as a plasma creatinine value higher than 110 µmol/L or a creatinine clearance lower than 50 ml/min per 1.73m^2), medications interacting with bone and mineral metabolism (bisphosphonates, lithium, loop diuretics or thiazides, corticosteroids), an associated disease (such as a progressive endocrine disorder,

neoplasia, or granulomatosis), or because they did not follow a low-calcium diet on the day before the investigation (Maruani et al, 2003). It is worth mentioning at this point, that the definition they used is not absolutely consistent with the one mentioned in the previous section.

The rest of the patients (178) were divided into two groups. Thirty-four (34) patients with normocalcaemia (based on fasting iCa≤1.35 mmol/L) were compared to a group of hypercalcaemic patients (mean age 55 and 53 respectively). In 27 out of the 144 patients with high ionised calcium (1.36-1.86 mmol/L), the total serum calcium was normal. The NPHPT patients were referred because of nephrolithiasis (35%), hypercalciuria (18%), radiographic bone demineralization (18%), discovery of a parathyroid tumour during ultrasonography and high serum PTH level value (21%), chondrocalcinosis (6%), and hypophosphatemia (9%) (Maruani et al, 2003).

The researchers identified that patients with normal calcium levels had lower PTH levels than hypercalcaemic patients, but their values overlapped (75±19 vs 93±49 pg/ml respectively, P<0.001). Their urinary calcium excretion was also significantly lower than the hypercalcaemic and so was their 1,25 dihydroxyvitamin D. Out of the 178 patients, 94 were operated because they fulfilled the 1990 guidelines mentioned above. Out of them, 21 were NPHPT and 73 were PHPT. The NPHPT patients had a smaller size of the removed gland than the hypercalcaemic (Maruani et al, 2003).

An oral calcium load test was performed in almost all patients to study several determinants of iCa levels. The test was not performed in patients with iCa >1.60 mmol/L. The patients were consuming food free from dairy products and were also given calcium-free water on the day before the test and were then fasted overnight. On the day of the test, they were given sufficient water to produce adequate urine

samples. An oral load of 1gr of elemental calcium was given, together with milk, after doing a 2-hour baseline urine test. Urine collected 90 minutes after this was discarded and a repeat 2-hour urine test was performed later. Halfway through the test, a blood measurement was performed without a tourniquet. Both the PHPT and NPHPT patients who had the test, showed an increase in ionised calcium with a slight (24% and 25% respectively) decrease of their PTH (Maruani et al, 2003).

As mentioned before, there was an overlap in the subjects' PTH values at baseline, which suggested that a given level of rise in PTH could induce hypercalcaemia in some patients but not all. In order to try and give an answer to this question, the researchers matched the 34 normocalcaemic with an equal number of hypercalcaemic patients. The matching was based on their age (within 5 years), sex and PTH levels (within 10 pg/ml). During this study, the researchers found a lower urine calcium excretion (fasting urinary calcium/creatinine) and a lower index of tubular calcium reabsorption (TRCa/GFR) in NPHPT than PHPT patients, which, nevertheless, was still higher than the one calculated in normal subjects. They also found lower markers of bone turnover (osteocalcin and fasting deoxypyridinoline), lower 1,25(OH)D synthesis and higher values of renal phosphate threshold in the patients with NPHPT, findings consistent with both kidney and bone PTH resistance (Maruani et al, 2003).

Conclusively, this study showed that the maintenance of normal calcium in these patients can be partly explained by the lower PTH values. This was supported by the fact that the parathyroid tumour mass was lower in these patients. Another explanation could be the fact that NPHPT is the first form of this disease, as mentioned above. However, this was not supported by this study, as the diagnosis was made in a similar age, and although these patients were followed up to 76 months, no-one became hypercalcaemic. The problem was that not all the patients had higher levels of PTH,

as these levels overlapped between the two groups. Therefore, there has to be a different explanation for the presentation. The researchers found milder PTH-induced bone effects in these patients (net bone release assessed by UCa/UCr and lower bone turnover markers) and milder PTH-renal effects (normocalcaemic patients had lower ability to reabsorb calcium, lower ability to decrease phosphate reabsorption and lower ability to synthesize calcitriol). One explanation for this resistance was thought to be the presence of the N-terminally truncated PTH fragment, which could antagonise the effect of PTH if it would be higher in the normocalcaemic patients. However, the nephrogenous cAMP secretion was similar in the two matched groups, indicating that the amount of serum bioactive 1– 84 PTH was the same. Another argument, was that other molecules rather than just PTH could be affecting the calcium handling, like sodium levels, acid-base balance and magnesium concentrations. However, the levels of urinary sodium excretion, pH, bicarbonate and serum magnesium were similar. Another possible explanation for this presentation, was thought to be a difference in vitamin D levels (vitamin D deficiency in the normocalcaemic patients). However, the levels of 25(OH)D were similar between the groups (Maruani et al, 2003).

In this study, body mass index (BMI) was higher in the NPHPT group and that could explain a higher oestrogen production and therefore a degree of resistance to the effects of PTH. Unfortunately, oestrogen levels were not measured in this study. Previous studies showed that oestrogen can protect the bones from PTH-induced calcium absorption and that, by giving oestrogen to PHPT patients, the serum and urinary calcium and the bone turnover markers can be reduced. Therefore, the researchers of the above-mentioned study, argued that normocalcaemic patients may unmask their hypercalcaemia after menopause, due to the oestrogen deficiency observed then. Some women with a sufficient endogenous production of oestrogen

after menopause may remain normocalcaemic (Maruani et al, 2003). The problem with this hypothesis is the fact that NPHPT is mainly seen in postmenopausal oestrogen-deficient women (Cusano et al, 2013b).

Based on the hypothesis that NPHPT can be caused by PTH resistance, and the fact that the single nucleotide polymorphism (SNP) rs17251221 (A986S) in the CaSR has been linked to PTH resistance, researchers in Spain recently investigated the effect of this SNP to NPHPT patients. They recruited and prospectively studied 61 consecutive patients with NPHPT and asymptomatic PHPT from their Endocrinology and Nutrition Service (for NPHPT, n=41, 83% female, mean age 63 years; for PHPT, n=20, 80% female, mean age 66 years). These patients had a follow up of one year to check for the persistence of their laboratory investigations on at least two occasions. They checked ionised calcium levels in their patients and they also used the following exclusion criteria for all their patients: eGFR<60 ml/min, 25(OH)D<30 ng/ml, FHH, hypercalciuria (>250mg/24h in females and >300mg/24h in men), other metabolic diseases and medications (thiazides, bisphosphonates, lithium). All the women were postmenopausal. There were significantly higher levels of PTH, and bone turnover markers in the PHPT group and lower levels of 25(OH)D, serum phosphate, magnesium and phosphate tubular reabsorption. Their BMD results were similar. There were 38 patients (62.3%, n=24 NPHPT and n=14 PHPT) with the wild type genotype A986A, and 23 (36.7%, n=17 NPHPT and n=6 PHPT) S allele carriers (n=20 A986S or n=3 S986S) (Diaz-Soto et al, 2015).

Seventeen NPHPT patients were S allele carriers. In these patients, the S allele was associated with significant higher levels of serum intact PTH (P=0.024) when compared with the wild type ones. No other comparison reached significant difference. On the contrary, the S allele PHPT patients, were older, had lower PTH, ALP and

PINP values ($p<0.05$). After adjusting for factors like vitamin D, calcium, albumin, phosphate and GFR, the association of PTH with the genotype remained significant for the NPHPT group. All the other predictors were not significant. This was not found in PHPT patients, in which only serum calcium was independently predicting the PTH level and not the genotype. Therefore, PTH levels might be regulated by the S genotype in NPHPT, which could be acting as a resistance factor (Diaz-Soto et al, 2015).

The underlying pathology of this disease seems to be similar to PHPT. A few studies report that multiglandular disease is more common in NPHPT (Koumakis et al, 2013) but not all reach statistical significance (Gómez-Ramírez et al, 2019; Kiriakopoulos et al, 2018). Most studies report that the average adenoma weight was lower in the NPHPT group compared to the PHPT group of patients (Kiriakopoulos et al, 2018; Koumakis et al, 2013; Maruani et al, 2003).

1.2.3.4 Prevalence

Due to the different definitions and methodologies used in publications on NPHPT, it is difficult to draw conclusions on the prevalence of this disease. The problem is that not all causes of secondary hyperparathyroidism were always excluded, thus the reported prevalence may be overestimated. Moreover, some of these studies did not test the persistence of the high PTH and normal calcium levels before evaluating the epidemiology. Finally, these reports include different age/gender specific populations. The prevalence of the disease in the literature varies between 0.1 and 8.9% (Pawlowska & Cusano, 2015) (Figure 1-13).

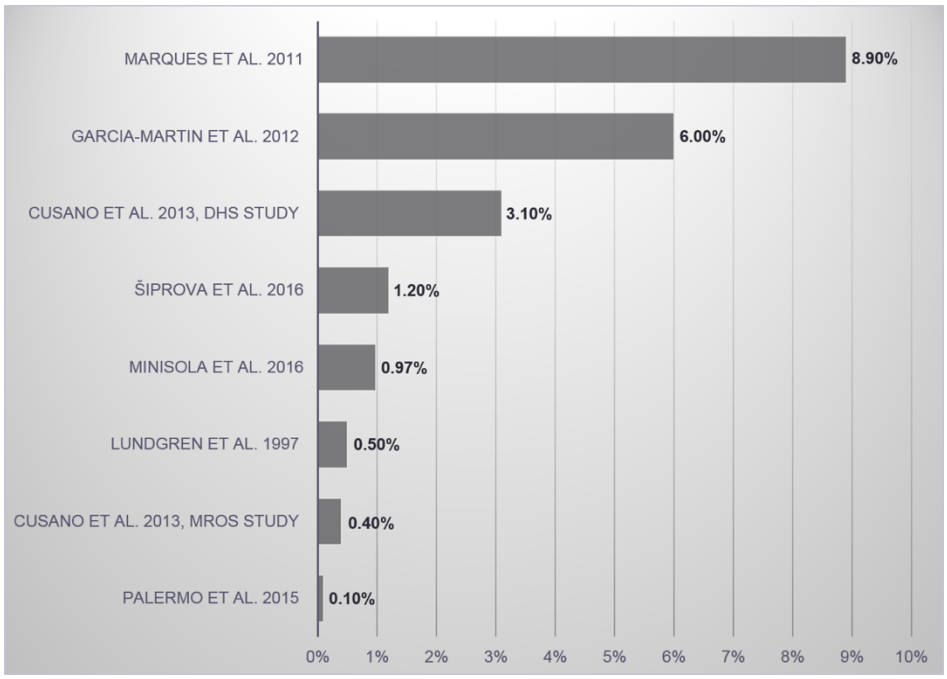


Figure 1-13: Prevalence of normocalcaemic hyperparathyroidism according to different publications

The issue of how the different definitions affect the prevalence reported, is specifically stressed in a recent publication. In this study, the researchers prospectively recruited adults ≥18 years who would be submitted to thyroidectomy due to nodular disease. They excluded patients who had an ultrasound due to PHPT, patients with a history of nephrolithiasis, nephrocalcinosis and pathological fracture, personal or family history of multiple endocrine neoplasia or diagnosis of medullary thyroid cancer. Their total cohort was 676 patients. Then, they estimated the prevalence of NPHPT based on the following criteria: normal adjusted and ionised calcium and high PTH, confirmed at two measurements, 25(OH)D ≥ 20 ng/dl, eGFR ≥ 40 ml/min/1.73m^2. They excluded people on diuretics, lithium, bisphosphonates, denosumab, recombinant PTH, corticosteroids, patients with primary aldosteronism, suspicion or known diagnosis of malabsorption, hyperphosphatemia, calcium/urinary creatine ratio ≥ 0.25, or thyroid dysfunction. They also screened for coeliac disease and excluded patients with positive antibodies. They found 46 (6.8%) patients with NPHPT. However, out of them

only 8.7% had altered parathyroid glands (adenoma) during bilateral gland exploration (0.6% of the cohort). They then calculated the prevalence based on different criteria on vitamin D deficiency and kidney function. They first used cut-offs of 20 ng/dl and 60 ml/min/1.73m^2 respectively. In this occasion, the prevalence was 4.4%, with confirmed pathology in 13.3%. When the cut-offs were 30 ng/dl and 40 ml/min/1.73m^2, the prevalence was 1.8%, with confirmed pathology of 33.3%. Finally, when using 30 ng/dl and 60 ml/min/1.73m^2, the prevalence was 0.74%, with confirmed pathology of 80% (Rosário & Calsolari, 2019).

In summary, NPHPT seems to be affecting the female gender more, and it is mostly observed after menopause, mainly between 60 and 70 years of age (Figure 1-14) (Cusano et al, 2013b; Siprova et al, 2016).

In surgical series, the prevalence on NPHPT amongst the patients that undergo parathyroidectomy, varies, and is reported to be approximately 10 to 20% (Gómez-Ramírez et al, 2019; Lim et al, 2017; Pandian et al, 2019; Pierreux et al, 2018; Sho et al, 2019; Yu et al, 2019).

More details on the prevalence are given in the subsequent chapter on NPHPT.

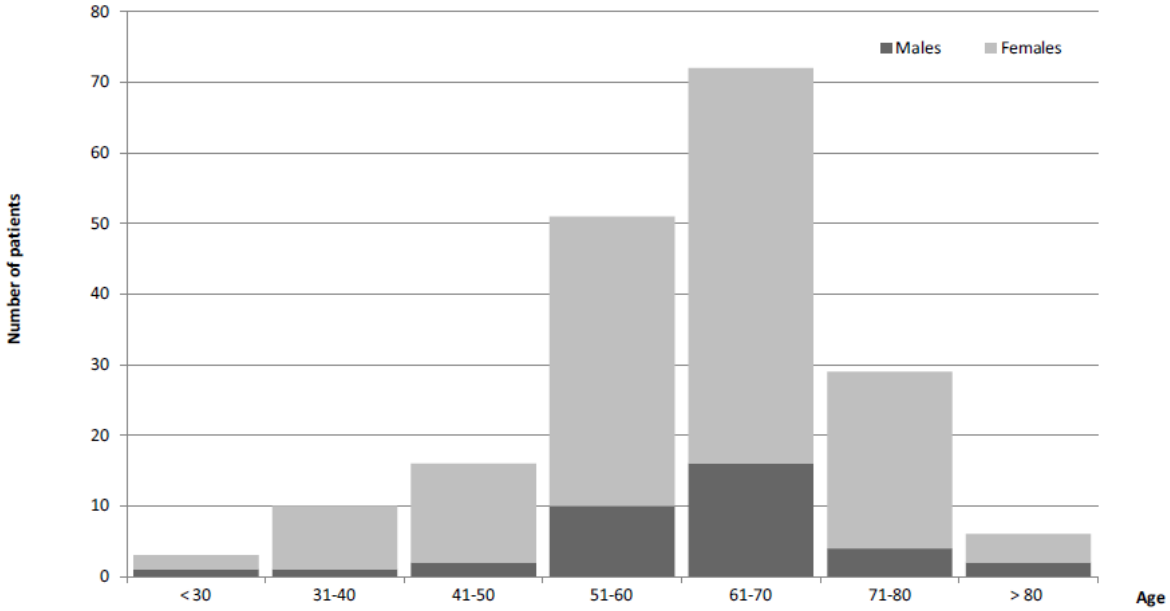


Figure 1-14: Age and gender distribution in a study of patients with normocalcaemic hyperparathyroidism

Reprinted from Endocrine Practice 2016 Vol 22, Šiprová H, Fryšák Z, Souček M., Primary hyperparathyroidism, with a focus on management of the normocalcemic form. To treat or not to treat, Pages 294-301, Copyright 2016, with permission from the American Association of Clinical Endocrinologists

1.2.3.5 Clinical presentation

NPHPT is usually encountered during the evaluation of secondary osteoporosis and has been linked to consequences known to affect patients with primary hyperparathyroidism (accelerated bone loss, kidney stones, higher levels of blood pressure, higher levels of fasting glucose and higher pre-existing glucose intolerance), but there is no clear evidence of a relationship. Data is mainly available from referral centres. There are limited studies comparing NPHPT patients to controls and the definition of NPHPT is not always the same in these studies. These results are

summarised below, but they should be treated with caution. More details can be seen in the tables in the Appendix.

1.2.3.5.1 Effects on skeleton and kidneys

Lowe et al identified 37 (95% female) NPHPT patients (although iCa was not identified for all), that were referred to the Metabolic Bone Disease Unit at Columbia University Medical Centre between 1998 and 2005. The presenting symptom in 57% of them was osteoporosis (mainly observed at the spine and hip rather than distal radius), while 11% had recent fragility fractures and 14% nephrolithiasis. They excluded cases with 25(OH)D<20 ng/ml, eGFR<40 ml/min/1.73m^2, liver disease, hypercalciuria >350mg/24h, thiazide or lithium use, metabolic bone diseases (Lowe et al, 2007). The problem with this study is that it is not clear whether they excluded patients on bisphosphonates or loop acting diuretics and it is not clear whether they checked the persistence of their laboratory measurements. The eGFR level was lower than the one described in the definition.

Comparing NPHPT to PHPT: A few years after the study described above, a group of researchers from an endocrine centre reported an 18.2% prevalence of nephrolithiasis and a 15.2% history of fractures in 33 normocalcaemic patients. The frequency of these complications was also studied in a group of hypercalcaemic subjects (18.9% and 10.8% respectively) and there was no statistically significant difference. The distal radius BMD was preserved compared to other sites in the NPHPT group, as reported by Lowe et al (Amaral et al, 2012). It is not clear whether persistence was checked.

The absence of statistically significant differences in the frequencies of nephrolithiasis, osteopenia and osteoporosis between NPHPT and PHPT patients was also confirmed in more recent publications (Pierreux et al, 2018; Tuna et al, 2015). Another centre in Spain, recruited 26 normocalcaemic and 16 hypercalcaemic patients and there was no significant difference in lumbar spine, femoral neck and total hip BMD between the two groups. However, they found that vitamin D receptor (VDR) polymorphisms were associated with differences in BMD measurement only in NPHPT patients. Homozygous NPHPT patients for the rs1544410 GG polymorphism and the rs731236 TT polymorphism, had lower hip BMD (Castrillon et al, 2015).

When comparing NPHPT patients to controls, as performed by some researchers in 2011, the prevalence of kidney stones reported in the subjects' medical files, was significantly higher in the normocalcaemic group (28.6% vs 0.7%, $P<0.001$), while there was not a significant difference observed in skeletal abnormalities (Marques et al, 2011). Another study showed similar percentages of nephrolithiasis (Temizkan et al, 2015). Analysis of the data from the Osteoporotic Fractures in Men Study (MrOS), also failed to prove significant differences in lumbar spine and femoral neck BMDs between NPHPT patients and controls (Cusano et al, 2013a).

As mentioned before, the majority of the studies were performed in referral centres, thus the prevalence of skeletal and renal abnormalities is expected to be higher. Only one study has been performed in the normal population. This was a prospective study in a cohort of 100 healthy postmenopausal women (age 56±3 years). NPHPT was defined as normal adjusted calcium and high PTH, with 25(OH)D>30 ng/ml, normal

renal function (creatinine clearance>70 ml/min/1.73m^2). In total 6 participants (6%) were identified. The bone turnover markers and bone mass assessed by quantitative ultrasound were normal (Garcia-Martin et al, 2012).

1.2.3.5.2 Cardiovascular risk factors

1.2.3.5.2.1 Glucose and lipid metabolism

The first study identifying possible cardiovascular risks in NPHPT was published in 2006; the researchers compared 30 postmenopausal women having both normal calcium and high PTH (study group) with 30 age-matched controls with similar levels of exercise and smoking. Higher levels of fasting glucose and a worse lipid profile (lower HDL, higher VLDL and triglycerides, higher LDL/HDL ratio) was observed in the study group. However, one of the main limitations of this study was the absence of vitamin D measurement (Hagstrom et al, 2006).

A few years later, the glucose metabolism of NPHPT patients and controls was evaluated in three case-control studies using an OGTT test and the homeostatic model assessment for insulin resistance (HOMA-IR). There was no statistically significant difference between the two groups (Cakir et al, 2012; Tassone et al, 2013; Temizkan et al, 2015). Moreover, the prevalence of DM and IGT was not significantly different between them (Tassone et al, 2013). Two of these studies also evaluated lipid levels and found no statistically significant difference (Cakir et al, 2012; Temizkan et al, 2015).

More recent publications though performed in Turkey, started to question these findings again. One study found higher levels of fasting glucose and higher pre-existing glucose intolerance (IGT/DM) in NPHPT patients compared to controls. These

levels were similar when comparing normocalcaemic and hypercalcaemic hyperparathyroid patients. Moreover, a positive correlation of both calcium and PTH to fasting insulin and HOMA-IR was identified. There was no statistically significant difference in lipid levels, although there was a higher usage of antilipidemics in the hyperparathyroid groups compared to controls. PTH was positively correlated to triglyceride levels (Yener Ozturk et al, 2015).

A more recent one found the prevalence of diabetes, dyslipidaemia, hypertension and insulin resistance to be similarly increased in the PHPT and NPHPT groups ($p > 0.05$) compared with the controls ($p < 0.05$). Moreover, glucose metabolism (glucose, insulin, HOMA-IR) and lipid profiles (total cholesterol, LDL, triglycerides) were similarly increased in the PHPT and NPHPT groups ($p > 0.05$) compared with the controls ($p < 0.05$). The levels of smoking and obesity were similar (Beysel et al, 2019).

Finally, one publication identified higher low-density lipoprotein (LDL) levels to a group of 23 patients (87% females, aged 54±11.4 years) compared to hypercalcaemic patients. These patients also had higher TSH values and that could explain the difference in LDL (Tuna et al, 2015).

1.2.3.5.2.2 Blood pressure and other cardiovascular risks

Increased systolic and diastolic blood pressure values were found in Chinese NPHPT patients when compared to controls matched for age, gender, calcium, BMI, glucose and lipid levels. This study's main limitations were the absence of ionised calcium measurement and the fact that the researchers did not evaluate smoking, alcohol consumption, physical activity or special diets in their subjects, factors that could affect blood pressure levels (Chen et al, 2015). These findings were not confirmed by Yener

Ozturk et al, although higher prevalence of pre-existing hypertension and use of anti-hypertensive drugs was observed in the NPHPT and PHPT groups compared to controls (Yener Ozturk et al, 2015). Absence of any statistically significant differences in blood pressure levels between NPHPT and age and BMI-matched controls, was reported recently in a separate centre in Turkey (Temizkan et al, 2015).

Similar prevalence of hypertension between NPHPT and PHPT groups, was observed in three further studies (Beysel et al, 2019; Tordjman et al, 2010; Tuna et al, 2015). In one of these studies, the levels of both systolic and diastolic blood pressure in PHPT and NPHPT were statistically higher than in controls (Beysel et al, 2019). In another study, NPHPT patients were found to have lower incidence of ischemic heart disease (IHD) and/or cerebrovascular accidents (CVA) compared to hypercalcaemic ones. Moreover, this study evaluated some non-invasive arterial stiffness parameters and found no differences between the 3 groups (NPHPT, PHPT, controls) (Tordjman et al, 2010).

1.2.3.5.3 Other features

The authors of a recent study on NPHPT, reported that patients with this disorder have less frequent symptoms (fatigue, indigestion, mood disorders, musculoskeletal pain) than PHPT ones (23.5% vs 71%, $P<0.001$). Furthermore, 76% of the 187 patients studied, had another endocrine disorder, mainly originating in the thyroid (Siprova et al, 2016).

1.2.3.6 Natural history

The problem with the study of the natural history of this disease, is that the persistence of the laboratory abnormalities is not always tested at baseline, and so the result of subsequent hypercalcaemia on follow-up should be treated with caution. The results from different studies performed on the natural history of calcium, can be seen in the subsequent chapter on NPHPT.

After a follow-up of 3.1±0.3 years, Lowe et al identified a patient with a new presenting kidney stone, one with a fracture and 11% with new developed osteoporosis. Two patients developed marked hypercalciuria (≥400mg/24h). Moreover, BMD decreased more than 5% at one or more skeletal sites in 43% of the patients and more than 10% in 20% , with the hip and forearm being the more severely affected. The magnitude of BMD change did not correlate with the PTH levels (Lowe et al, 2007).

When studying the natural history of calcium, seven patients (19%) developed hypercalcaemia. None of the patients that became hypercalcaemic met the criterion of surgery for elevation of calcium more than 1 mg/dl above the normal range. The patients who became hypercalcaemic were older and had higher serum and urine calcium at baseline (Lowe et al, 2007). Progression to hypercalcaemia was also confirmed in some patients in other studies. Higher calcium levels at baseline were considered to predict progression to hypercalcaemia in one of these studies (19% progression). Lower phosphate level was also a predictive factor (Siprova et al, 2016).

Silverberg et al found hypercalcaemia in three patients (14%) after one year of follow up; these patients had higher PTH levels compared to the ones that remained normocalcaemic. The patients of this study had confirmed persistence of laboratory results at baseline (Silverberg & Bilezikian, 2003).

On the other hand, there are several publications failing to prove a progression to hypercalcaemia (Ayturk et al, 2006; Garcia-Martin et al, 2012; Hagstrom et al, 2006; Tordjman et al, 2004; Tordjman et al, 2010).

1.2.3.7 Management

1.2.3.7.1 Monitoring

It has been proposed that calcium and PTH should be tested every year, while a BMD check should be performed every 1-2 years. If there is a progression to hypercalcaemia, patients should be treated according to the guidelines on asymptomatic primary hyperparathyroidism. If calcium remains normal, but an accelerated bone loss or a fracture is observed, or if the patient develops a kidney stone or nephrocalcinosis, then surgery should be an option (Figure 1-15) (Bilezikian et al, 2014).

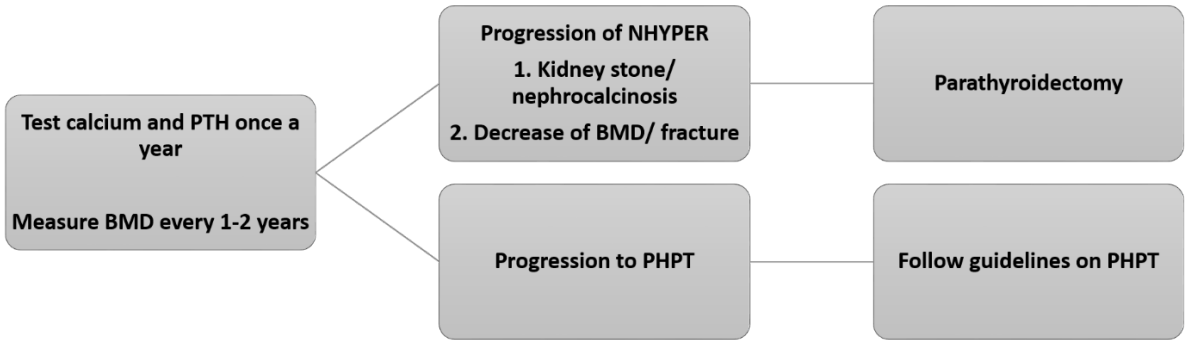


Figure 1-15: Monitoring of patients with normocalcaemic hyperparathyroidism as advised at the Fourth International Workshop on Asymptomatic Primary Hyperparathyroidism

Figure adapted from the original (Bilezikian et al, 2014)

There have been some publications questioning possible treatment effects, using either surgical or medical treatments. These are described in the next sections.

1.2.3.7.2 Surgical management

The number of publications reporting surgical outcomes on NPHPT have increased dramatically over the last few years. The results are summarised here and more details can be found in the tables for NPHPT in the Appendix.

1.2.3.7.2.1 Results

1.2.3.7.2.1.1 Effect on the skeletal and kidney abnormalities

One study evaluated 413 consecutive patients referred to a Metabolic Bone Centre in Paris between 2008-2010. Amongst them, 193 had PHPT and 220 had secondary hyperparathyroidism. They looked at the data of 168 patients that had parathyroidectomy by the same surgeon and then excluded any patients that had no BMD follow up (n=98), patients that had hypercalcaemia after surgery (n=11) and had normal parathyroid at the histological analysis (n=2). In total, they studied 60 patients and divided them into two groups: 39 were in the normocalcaemic group and 21 were in the hypercalcaemic one. The diagnosis of PHPT was based on elevated or inappropriately normal PTH with increased adjusted calcium or ionised calcium. NPHPT was defined as normal adjusted calcium and high PTH, after excluding causes of secondary hyperparathyroidism like: 25(OH)D<20 ng/ml, renal impairment (GFR<40 ml/min/1.73m^2), bisphosphonate, thiazides, anticonvulsants, lithium, gastrointestinal diseases related to malabsorption and liver disease. In those with

hypercalciuria, a thiazide diuretic test was performed. An oral calcium load test was performed in all the patients and NPHPT was defined as total calcium and/or ionised calcium increasing to supranormal values with only a minimal reduction in PTH. Patients that did not have an increase of more than 1.42 mmol/L of ionised calcium, were also given a 20-minute infusion of 2mg/kg elemental calcium (Koumakis et al, 2013).

In the normocalcaemic group (only 41% had normal iCa), there was a significant increase of the BMD both at the spine and hip (+2.3±5.0%, P=0.016 and +1.9±5.7%, P=0.048 respectively) compared to baseline. Similar increases were seen in the PHPT group. The only difference was a drop in the distal 1/3 of radius BMD in the NPHPT group (-1.5±3.5%, P=0.02). On the contrary, BMD remained stable in the radius in the PHPT group. This study reported that there were more cases of multiple adenomas and hyperplasia in the normocalcaemic group compared to the hypercalcaemic one (P=0.04). The weight of the adenomas was also lower (Koumakis et al, 2013).

Due to the fact that this study identified a significant decrease in the BMD measured at the distal 1/3 of radius in NPHPT patients, the researchers conducted a further study on the same group of patients, in order to evaluate the benefit of surgery in an individual patient. They only included 36 of the patients that had BMDs at all sites of interest and considered BMD gain significant for an individual patient if it was ≥0.030 g/cm^2 at any site, without any loss of BMD of ≥0.030 g/cm^2 at another site during the same period of time. A year after the recruitment, 44.4% normocalcaemic patients had an individual BMD gain in at least one site (spine, hip, forearm), without any loss at any other site, compared to 73.7% in the hypercalcaemic ones (p=0.049). There was no difference statistically when comparing the gain between the patients with normal or elevated ionised calcium within the normocalcaemic group. Pre-surgery level of

ALP above the median was identified as a predictive factor of BMD gain after the surgery. The researchers conclude by saying that the short-term decrease in BMD at the radius does not reflect the global change in BMD and that surgery should be an option to these patients (Koumakis et al, 2014).

Promising results after surgery also come from another study performed a few years later. In this study, all patients that had a parathyroidectomy in a single centre between 2006 and 2016 were evaluated. They only included patients with post-surgery biochemical data available more than six months after surgery. They also had data on some patients on BMD results performed more than two years after surgery. In total, they included 71 patients with NPHPT, defined as normal calcium and high PTH (only patients with peak calcium within the reference range within one year prior to surgery). They excluded patients with 25(OH)D<20 nmol/L and eGFR<60 ml/min/1.73m^2. They found that persistently elevated PTH was common, occurring in 46.5% of patients after surgery. BMD results (spine, hip, femur neck) were available to 38 of them, showing a BMD improvement of 5.6% ($p < 0.01$) in patients with normal PTH after surgery. No significant change was observed in patients with persistently elevated PTH after surgery (- 2.2%, $p = 0.47$) (Sho et al, 2019). At this point, it has to be pointed out that some of the patients had 25(OH)D<20 nmol/L, so probably not all causes of secondary hyperparathyroidism were excluded.

In terms of kidney effects, one recent study followed up patients for 72.9 ± 46.8 months after surgery. Ten patients with prior kidney stones had a follow up. Four patients (40%) had no evidence of kidney stones. One patient had persistence of microlithiasis without symptoms. The rest had stable results (Traini et al, 2018).

1.2.3.7.2.1.2 Effect on the cardiovascular abnormalities

As far as the effect of surgery on metabolic abnormalities is concerned, no conclusions can be made from the current literature. A publication in 2006, showed that patients had an improvement of their lipid profile (total and LDL cholesterol, LDL/HDL ratio) after surgery, but also reported an increase of glucose and urate. The specific study though, did not evaluate vitamin D levels (Hagstrom et al, 2006). More recently, a publication from Turkey showed a reduction in both systolic and diastolic blood pressure, total cholesterol and HOMA-IR in NPHPT and PHPT patients six months after parathyroidectomy. The cardiovascular risk score also decreased (Beysel et al, 2019).

1.2.3.7.2.1.3 Other effects

Recently, there was a study published investigating the effect of parathyroidectomy on cognitive function in patients with NPHPT followed up at Columbia University in New York; there was no improvement observed six months after surgery (Liu et al, 2019).

The cure rate is reported to be similar in the PHPT and NPHPT group (97.53% vs 91.43% respectively, p=0.108). The incidence of temporary hypocalcaemia also seems to be similar (Yu et al, 2019). One group reported a percentage of 2% of permanent hypocalcaemia (Traini et al, 2018).

In terms of normalisation of postoperative PTH, a recent study showed that persistently elevated PTH was common, occurring in 46.5% of patients after surgery. This increase was found at a median time of 7.7 months. Preoperative PTH levels

>100 pg/ml were related to persistently elevated levels after surgery, However, this study only excluded patients based on 25(OH)D<20 nmol/L (Sho et al, 2019). In the study by Koumakis et al, three months after surgery, 21% of the operated NPHPT patients had elevated PTH levels but these were found to be related to vitamin D deficiency and were normalised after supplementation (Koumakis et al, 2013). Another group divided the patients to the ones having normal ionised calcium prior to surgery and the ones having high. Amongst the group with normal ionised calcium, 25% still had elevated PTH after surgery compared to 8% with elevated ionised calcium; the difference was not statistically significant (Wade et al, 2012).

1.2.3.7.2.2 Histology

The underlying pathology of this disease seems to be similar to PHPT. A few studies report that multiglandular disease is more common in NPHPT (Koumakis et al, 2013; Lim et al, 2017; Pandian et al, 2019; Traini et al, 2018) but not all reach statistical significance (Gómez-Ramírez et al, 2019; Kiriakopoulos et al, 2018; Yu et al, 2019). This means, that NPHPT patients are be more likely to have bilateral exploration (53.9 vs 44.2%, in the PHPT group, $p < 0.05$) (Traini et al, 2018).

According to one of these studies, patients with NPHPT were over eight times as likely to have multiglandular disease (odds ratio 8.17, 95% confidence interval 4.49 to 14.83) (Lim et al, 2017). However, the criteria used for NPHPT were unclear in this study, but it seems that they included patients with eGFR<60 ml/min/1.73m^2, lithium use and/or 25(OH)D<30 ng/ml in the NPHPT group, which could theoretically explain the findings. The study by Pandian et al also included patients with low vitamin D, and did not have

data on kidney function, so probably several of these patients had secondary hyperparathyroidism (Pandian et al, 2019).

Most studies report that the average adenoma weight was lower in the NPHPT group compared to the PHPT group of patients (Kiriakopoulos et al, 2018; Koumakis et al, 2013; Maruani et al, 2003). To my knowledge, no study has reported parathyroid cancer in NPHPT.

1.2.3.7.2.3 Presurgical assessment: issues to be considered

1.2.3.7.2.3.1 Ionised calcium

A recent publication from a surgical department in Wisconsin, mentions the importance of measuring ionised calcium before surgery. The authors of this manuscript retrospectively evaluated records from 771 patients who had parathyroidectomy between 1999 and 2008 and defined NPHPT as patients having normal calcium levels during the three months prior to surgery. Patients with recurrent, familial, secondary or tertiary hyperparathyroidism were excluded, although the criteria for exclusions are not clear. Out of these patients, 93 (12%) had normal total calcium and 58 of them also had available ionised calcium. Amongst these, 50 (86%) had elevated ionised calcium and only eight (14%) had both total and ionised calcium within the normal range. When comparing the two last groups (elevated and normal ionised calcium), there was no difference in age and gender. The prevalence of nephrolithiasis (24% vs 24% respectively), osteoporosis (18% vs 25%), bone fracture (12% vs 13%) were similar ($p>0.05$). The levels of PTH, 24-urine calcium and vitamin D were also similar (Wade et al, 2012). At this point, it has to be pointed out that some

of the patients had 25(OH)D<20 nmol/L, so probably not all causes of secondary hyperparathyroidism were excluded.

The results of the surgery did not differ between the groups (similar number of single gland and multiple gland disease, similar gland weight) and the intraoperative PTH measurement was successful in the same number of patients. After a median follow up of 13 months, patients from both groups had similar levels of total and ionised calcium, however, there was a significantly higher level of PTH in the normal ionised calcium group (Wade et al, 2012).

The importance of ionised calcium was also mentioned in a more recent publication, where 104 patients (77.0% female, mean age 60.5) were enrolled after being referred for parathyroidectomy between 2015 and 2017 in an Endocrine Surgery department in Madrid. When considering adjusted calcium levels, 45 (43.3%) were classified as NPHPT. When assessing ionised calcium, 64% of the patients who were initially classified as NPHPT had high ionised calcium levels, leaving only 16 to study further in the NPHPT group (Gómez-Ramírez et al, 2019).

1.2.3.7.2.3.2 Free 25(OH)D

Another issue that came up recently was the fact that NPHPT patients seem to have lower free 25(OH)D than healthy controls and thus may actually have secondary hyperparathyroidism. This study recruited 10 NPHPT patients based on the following criteria: normal calcium level, elevated PTH and 25(OH)D 30-40 ng/ml, having normal liver and renal function. They excluded alcoholics or chronic smokers, people on medications like contraceptive pills, proton pump inhibitors, diuretics, or any medications that affect calcium or bone metabolism, and patients with evidence of

bone disease in the past year. At the same time, they included 20 age, sex, and BMI-matched subjects with normal PTH in their control group. All subjects were on vitamin D supplements (400 IU). Although the levels of 25(OH)D were similar between the groups, the free 25(OH)D levels were 20% lower in the NPHPT group ($p < 0.05$). PTH was inversely correlated with free 25(OH)D but not with total 25(OH)D (Wang et al, 2019).

1.2.3.7.2.4 Preoperative localisation

The localisation of the parathyroid pathology was reported to be different in NPHPT and PHPT patients by a study in Czech Republic. NPHPT patients who ultimately evolved to hypercalcaemia had an adenoma identified in 4% of the cases when they were at their normocalcaemic state, while an adenoma was identified in 73% of the cases when they were in their hypercalcaemic state ($p=0.001$) (Siprova et al, 2016).

Both sestamibi and neck ultrasound were found to underperform in NPHPT compared to PHPT in a study in Madrid, but the difference was not statistically significant (Tc-sestamibi: sensitivity in PHPT 81% vs 56.2% in NPHPT; ultrasound sensitivity in PHPT 50.6% vs 31.2% in NPHPT) (Gómez-Ramírez et al, 2019). A recent analysis showed that there is a higher probability of accordance between the two imaging techniques the higher adenoma size and lower accordance in multigland disease, although the difference was not statistically significant for the second comparison (Kiriakopoulos et al, 2018).

The fact that NPHPT has been reported to be correlated to multigland disease, could complicate the localisation imaging studies. In one study, both the sestamibi and

ultrasound together, failed in detecting all the diseased glands in 85.0% NPHPT patients and in 79.5% PHPT (Traini et al, 2018).

A recent study in Brazil evaluated 18 patients with surgically proven PHPT and a histological diagnosis of adenoma. Eight had NPHPT (mean age 55.1 ±14.4 years, 83% female). They all underwent three preoperative imaging techniques [ultrasound, Tc-99-sestamibi scintigraphy and 4-dimensional computed tomography (4D CT)]. The sensitivity of all the techniques was lower for the NPHPT group compared to the PHPT group. Four-dimensional computed tomography performed better for NPHPT. The results were as follows: ultrasound 22% vs 58.3%, scintigraphy 11.1% vs 75%, and 4-dimensional computed tomography 55.6% vs 75% (Cunha-Bezerra et al, 2018).

PET/CT with 18F-Fluorocholine (18F-FCH) also seems to be having positive results in NPHPT patients. Researchers in Italy enrolled 34 consecutive patients with PHPT and amongst them, 7 had NPHPT. The detection rates in NPHPT and PHPT were 57 and 70% for ultrasound respectively, 70 and 71% for 18F-FCH-PET/CT, and 0% and 18% for sestamibi (Bossert et al, 2019).

1.2.3.7.3 Pharmacological management

There are limited data on the medical approach of NPHPT. A prospective open label randomized study was published recently, evaluating possible pharmacological treatment for NPHPT, using a combination of weekly oral alendronate (70mg) and colecalciferol (2800IU) in 15 NPHPT postmenopausal women (treatment group) and comparing them to the same number of patients treated only with colecalciferol (control group). The inclusion criteria were: postmenopausal status of more than five years, osteoporosis, high PTH or in the upper third of the reference range and normal

adjusted calcium, normal vitD (≥30 ng/ml). The researchers excluded patients with secondary hyperparathyroidism, concurrent systematic illness, thyroid disease, hepatic or renal dysfunction, disorders known to influence BMD, medication use (bisphosphonate, oestrogen, vitamin D or calcium supplements, drugs to interfere with bone metabolism during the last 12 months), reported family or personal history of recurrent kidney stone disease (Cesareo et al, 2015). Ionised calcium was not included in the definition criteria and it is not clear if the researchers looked for persistence.

The two groups had no differences at baseline in terms of PTH, calcium, phosphate, vitD, osteocalcin, CTX and 24h urine calcium. After one year of treatment, there was a significant BMD increase in the treatment group, mainly observed at the lumbar spine (mean increase 4.7%) than the hip (mean increase 4%). On the other hand, there was a significant decrease of the BMD at all sites in the control group. The radius BMD was not evaluated. Moreover, there was a significant decrease in bone turnover markers (osteocalcin and urinary CTX) in the treatment group observed after 3 months of treatment; the values remained reduced when retested at 6 months (not tested at 12 months). The two groups did not show any statistically significant differences in serum and urinary calcium levels when tested at 3-month intervals, thus indicating that it was safe to treat with vitamin D (Cesareo et al, 2015). The results of this study are not really surprising. Giving alendronate would increase BMD in all older women with osteoporosis, not just with NPHPT. Including a control group with osteoporosis but without NPHPT would had been more helpful.

There was another prospective randomized pilot study published in 2014, testing the effect of cinacalcet in normocalcaemic (n=6) and hypercalcaemic (n=4) hyperparathyroid patients with active kidney stone disease (≥2 stones in the past 2

years). The normocalcaemic patients were defined based on their consistently high PTH, after excluding causes of secondary hyperparathyroidism like 25(OH)D < 20 ng/ml, eGFR<50 ml/min, medication (thiazide, lithium), gastrointestinal disorders related with malabsorption, idiopathic hypercalciuria (>300 mg/24h in men, >250 mg/24h in women or greater than 4mg/kg of body weight for both genders, in the absence of other causes of hypercalciuria). For patients with a urinary calcium of >4mg/kg of body weight, they performed a thiazide test; they treated the patients for three months with a thiazide diuretic like hydrochlorothiazide 12.5 mg twice a day and observed that in these patients, PTH remained high and even elevated further despite the decrease in urinary calcium and the elevation of the serum calcium. This confirmed that hypercalciuria was not the cause of the elevated PTH (Brardi et al, 2015).

The included patients were treated with either potassium citrate and allopurinol, or cinacalcet for 10 months (dose titrated to lower PTH to normal limits and maintain normal calcium). At the same time, they followed the same dietary (normal calcium intake (800-1000mg daily) and hydration instructions (> 2 litres). By the end of the treatment period, there was a reduction in the number and diameter of the stones in patients treated with cinacalcet, both in the normocalcaemic and hypercalcaemic group (Brardi et al, 2015). The small number of patients included in this study is a major limitation; it is difficult to draw conclusions.

1.2.4 Hypoparathyroidism

On the other side of the spectrum of calcium related disorders, hypoparathyroidism is a rare disorder, characterised by low levels of calcium due to a low PTH secretion. Hypoparathyroidism was characterised as an orphan disease by the European Commission in January 2014 (Bollerslev et al, 2015).

1.2.4.1 Aetiology and prevalence

The most common cause is acquired hypoparathyroidism, mainly seen after anterior neck surgery resulting in the damage of the parathyroid glands (approximately 75% of cases). Chronic hypoparathyroidism after surgery, is defined as the one persistent six months postoperatively. In general, surgery can cause permanent hypoparathyroidism in 0.12 to 4.6% of cases (Bollerslev et al, 2015; Brandi et al, 2016).

Another cause is autoimmune hypoparathyroidism, which can be isolated or part of a polyglandular syndrome. Destruction of the parathyroids can also occur after radiation or infiltration by neoplastic or granulomatous tissue. Primary causes are rarer, and include several inherited syndromes summarised in Table 1-5 (Bollerslev et al, 2015; Brandi et al, 2016).

There are not many reports available regarding the prevalence of this disease and most of them use hospitalisation codes to identify patients, and so the prevalence is probably underestimated. The prevalence of this disorder was studied by a group in Denmark. They used data from the National Hospital Patient Registry in order to identify patients with non-surgical hypoparathyroidism. Using discharge codes, they identified 180 patients diagnosed with non-surgical hypoparathyroidism between 1997 and 2012 (prevalence 2.3/100000 inhabitants) (Underbjerg et al, 2015). Post-surgical

hypoparathyroidism had a prevalence of 22/100000 inhabitants (Underbjerg et al, 2013). Similar estimates come from the United States (Clarke et al, 2016).

1.2.4.2 Diagnosis

Hypoparathyroidism is characterised by undetectable or inappropriately low PTH along with hypocalcaemia (measured by albumin-adjusted calcium and ideally ionised calcium), confirmed on at least two occasions at least two weeks apart. Phosphate can be in the upper normal or elevated. Usually, 1,25-dihydroxyvitamin D and bone markers are low. Generally, urinary calcium is expected to be high, but due to the filtered calcium load being lower than normal, urinary excretion might be normal, especially before treatment. During the evaluation for hypoparathyroidism, magnesium also has to be assessed to exclude deficiency (Brandi et al, 2016). The renal function also has to be evaluated.

1.2.4.3 Clinical manifestations

The symptoms depend on whether hypocalcaemia developed acutely or not and on the absolute levels of calcium in the blood. In cases of surgery, where acute hypocalcaemia is often seen, dramatic symptoms can be present like seizures, laryngospasm, acute cardiomyopathy and heart failure due to decreased contractility (Abate & Clarke, 2016). Chronic hypocalcaemia on the other hand can be mild with non-specific symptoms and can result in multiple organ dysfunction with neurological, muscular and cardiac manifestations. Low calcium in combination with high phosphate, can result in high calcium-phosphate product, and can cause to soft tissue

calcifications (basal ganglia, stones and nephrocalcinosis, joints, eyes, skin, vasculature) (Brandi et al, 2016).

Classical clinical signs of hypoparathyroidism are Chvostek's sign (induction of facial muscle spasm by tapping the parotid gland over the facial nerve) and Trousseau's sign (inflation of a blood pressure cuff around the arm to greater than the systolic pressure, can cause carpopedal spasm) (Cooper & Gittoes, 2008).

Hypoparathyroidism is characterised by reduced bone turnover and increased bone mass, although whether this has an effect on fracture risk is really unknown (Abate & Clarke, 2016; Brandi et al, 2016). These patients when treated often become hypercalciuric and are at risk of renal calcium deposition (Brandi et al, 2016). A common complication in this disorder is renal insufficiency (41%). Mitchell et al reported stage 3-5 chronic kidney disease to be 2-to-17-fold greater compared to normal controls with 31% of the patients that had imaging, having renal calcifications (Mitchell et al, 2012).

Hypoparathyroidism was found to affect quality of life, with patients reporting symptoms of "brain fog", fatigue and higher incidence of psychiatric disorders like anxiety and depression (Abate & Clarke, 2016). In a study performed in Norway, they invited the patients to participate in the study by completing a questionnaire, and they found worse scores on the Short Form 36 (SF-36) and the Hospital Anxiety and Depression Scale (HADS) when compared to the normative population (Astor et al, 2016).

Other manifestations include increased risk of subcapsular cataract, cardiac arrhythmias and basal ganglia calcifications (reported in 52% of those imaged). The hospitalisation rates are also high (33%) (Mitchell et al, 2012).

The complications were recently described by a group in Denmark and surgical and non-surgical forms were distinguished. They compared the hypoparathyroidism patients identified, with a group of gender and year of birth controls to assess mortality and morbidity. In the non-surgical ones, the mortality was not found to be increased but, the patients were found to have increased risk for renal insufficiency (HR 6.01), cardiovascular disease (HR 1.91), seizures (HR 10.05), cataract (HR 4.21), neuropsychiatric diseases (HR 2.45), fractures in the upper extremities (HR 1.93) and infections (HR 194). They also showed that the patients had reduced risk of malignancy compared to controls (HR 0.44) (Underbjerg et al, 2015). In the patients with postsurgical hypoparathyroidism, they found increased risk of renal complications (HR 3.67), hospitalisation due to infections (HR 1.42) or seizures (HR 3.82) and an increased risk of depression and/or bipolar affective disorders (HR 1.99). There was not an increased risk of cardiovascular diseases or death and the risk of gastrointestinal cancers was lower (HR 1.99). The risk of cataract or any fracture was not increased, whereas the risk of fractures of the upper extremities was reduced (HR 0.69) (Underbjerg et al, 2013; 2014).

1.2.4.4 Treatment

1.2.4.4.1 Acute hypocalcaemia

This is a medical emergency and has to be treated. In general, calcium gluconate is preferred, because calcium chloride can cause local irritation when given intravenously. One or two 10 ml ampoules of 10% calcium gluconate, diluted in 50-100 ml of 5% dextrose, should be slowly infused over 10 minutes. If persistent, ten 10 ml ampoules of 10% calcium gluconate diluted in one litre of 5% dextrose or 0.9%

saline can be given at a rate of 50 ml/hour and adjusted accordingly. This should increase the calcium by 0.3-0.5 mmol/L over four to six hours (Cooper & Gittoes, 2008). In general, postsurgical hypocalcaemia can be managed with oral supplements.

1.2.4.4.2 Chronic hypocalcaemia

1.2.4.4.2.1 Conventional therapy

Treatment should be given to patients with adjusted calcium <2.0 mmol/L and to symptomatic patients with calcium between 2.0 mmol/L and the lower limit of the range. The goal of treatment is to prevent signs and symptoms of low calcium, to maintain calcium slightly above normal or slightly below the lower limit of normal and to avoid hypercalciuria. The serum calcium–phosphate product should be below 4.4 $mmol^2/L^2$. Dietary phosphate should be decreased (Bollerslev et al, 2015; Brandi et al, 2016).

Calcium carbonate is the most common form of calcium given (contains 40% elemental calcium). However, it needs an acidic environment in the stomach in order to be absorbed. Therefore, calcium citrate (20% elemental calcium) can be used in patients with achlorhydria, proton pump inhibitor use or constipation caused by calcium carbonate. Calcium should be used in 2 to 4 divided doses (Abate & Clarke, 2016).

Because of the impaired conversion of 25(OH)D to its active form, the active analogue of vitamin D (calcitriol) is used for treatment. Usually the dose varies between 0.25 and 2µg daily. Alphacalcidol can also be used alternatively (0.5-4.0 µg/day). Any changes in the dose should be adjusted every four to seven days. Patients should also

be given supplementation with ergocalciferol or colecalciferol (400-800 IU) because that can enhance the action of vitamin D. Vitamin D deficiency was associated with myopathy and neuromuscular complains (Abate & Clarke, 2016; Bollerslev et al, 2015; Cooper & Gittoes, 2008).

Thiazide diuretics along with low salt diet can also be used in cases with hypercalciuria, since they reduce the urine calcium. Patients on this treatment should be monitored for hypokalaemia and hypomagnesemia. Magnesium supplementation should also be given in patients with low magnesium. Phosphate diets and phosphate binders are considered in cases where phosphate is extremely high (>6.5mg/dL) (Bollerslev et al, 2015; Brandi et al, 2016).

1.2.4.4.2.2 PTH treatment

Recently (in 2015), the use of PTH (1-84) has been approved by the American Food and Drug Administration (FDA) for the treatment of patients with hypoparathyroidism, excluding the ones with autosomal dominant hypocalcaemia (ADH). Treatment should be considered in patients with not well controlled serum calcium, taking large doses of calcium or vitamin D supplementation (>2.5g calcium or >1.5μg calcitriol or >3.0μg of alphacalcidol), having renal complications (hypercalciuria, stone risk, nephrolithiasis, nephrocalcinosis, eGFR<60 ml/min/1.73m^2), having high phosphate or high calcium phosphate product (>55mg^2/dL^2 or 4.4 $mmol^2/L^2$). Moreover, malabsorption and reduced quality of life are also indications. The treatment should start by giving 50μg daily sc and a 50% reduction to the active form of vitamin D should be performed. The goal is to reduce the use of calcitriol, decrease the dose of calcium to 500mg daily and maintain serum calcium at the low-normal levels (Brandi et al, 2016).

1.2.4.5 Monitoring

Calcium, phosphate, magnesium and eGFR should monitored every three to six months. Twenty four hour urine collections for calcium and creatinine should be repeated yearly along with a GFR estimation. If there is a history of nephrolithiasis or nephrocalcinosis, renal imaging should be performed every five years in asymptomatic patients. Follow up should be done more frequently if symptoms develop. There are no guidelines regarding the testing on basal ganglia calcifications. Follow up of cataract will depend on baseline findings. BMD is not routinely measured (Bollerslev et al, 2015; Brandi et al, 2016).

Disorder	Gene; chromosomal abnormality Inheritance	Phenotype
Syndromic forms		
Autoimmune polyglandular syndrome type I (APS I)	AIRE (autoimmune regulator 1), chromosome 21q22.3 Sporadic, AR	Hypoparathyroidism, adrenal insufficiency, mucutaneous candidiasis. Other endocrinopathies: hypogonadism, diabetes mellitus type 1, hypothyroidism. Non-endocrine disorders: pernicious anaemia, vitiligo, alopecia
DiGeorge/CATCH-22	TBX1 (T-box 1); Microdeletions in 22q11 AD	Cardiac abnormality, abnormal facies, thymic hypoplasia (impaired T cell immunity and infections), cleft palate, hypocalcaemia (hypoparathyroidism 60%), with deletion in chromosome 22
CHARGE	CHD7 (chromodomain helicase DNA-binding protein 7); 8q12.1–q12.2, 7q21.11 AD	Coloboma of the eye, heart malformation, choanal atresia, retardation of growth and development, and genital and ear abnormalities, gonadotropin deficiency, anosmia
Hereditary deafness and renal dysplasia syndrome (HDR)	GATA3 (GATA-binding protein 3); 10p14 AD	Deafness, renal dysplasia
Kenny-Caffey syndrome 1, Sanjad-Sakati syndrome	TBCE (tubulin specific chaperone E); 1q42.3 AD, AR	Short stature, osteosclerosis, cortical thickening of long bones, delayed anterior fontanel closure, basal ganglia calcification, hyperopia
Kenny-Caffey syndrome 2	FAM111A (family with sequence similarity 111 member A), chromosome 11q12.1 AR	
Dubowich syndrome	Unknown AR	Microcephaly, abnormal facies, short stature, mental retardation
Bartter syndrome type 5	CaSR (3q21.1)	Hypokalaemia, metabolic alkalosis

	AD	
Nephropathy, nerve deafness	Unknown AD	
Nerve deafness without renal dysplasia	Unknown AD	
Kearns-Sayre syndrome	Mitochondrial defects Maternal	Muscle defect: ophthalmoplegia, proximal muscle weakness, bilateral pigmented retinopathy, cardiac conduction abnormality, cerebral ataxia, deafness, diabetes mellitus, growth hormone deficiency, short stature
Mitochondrial trifunctional protein deficiency syndrome (MTPDS)		
Mitochondrial encephalopathy, stroke like episodes and lactic acidosis (MELAS)		
Non-syndromic forms		
Isolated hypoparathyroidism	GCM2 (gliad cell missing gene), chromosome 6p24.2 AR	
	SOX3 (sry related HMG box), Xq26-q27 X-linked	Males with infantile seizures
	PTH, chromosome 11p15 AD, AR	
Autosomal dominant hypocalcaemia 1 (ADH)	CaSR activating mutations; 3q21.1 AD	Hypocalcaemia, low PTH, hypercalciuria, low Mg
ADH2	GNA11 (G protein subunit alpha 11); 10p13 AD	

Table 1-5: Inherited causes of hypoparathyroidism.

Adapted from original (Abate & Clarke, 2016). AD: autosomal dominant, AR: autosomal recessive; PTH: parathyroid hormone; CaSR: calcium-sensing receptor

1.2.5 Normocalcaemic hypoparathyroidism

Normocalcaemic hypoparathyroidism (NHYPO), is characterised by normal levels of calcium with persistent low levels of parathyroid hormone (PTH). There is little in current literature on this disease, with only two studies (reporting three different populations) published on its prevalence, whilst its natural history remains relatively unknown (Cusano et al, 2013a; Palermo et al, 2015). The prevalence in these studies is reported to be 1.1-2.4% at baseline. Two of these populations were then checked six and eight years later and only 0.6% and 0.09% respectively were still characterised as having NHYPO. None developed hypocalcaemic hypoparathyroidism. Serum calcium and PTH was only checked on two occasions in these studies, so the natural history of serum calcium is unclear. To our knowledge, no one has evaluated this before. Further information can be found in the relevant chapter on NHYPO.

Section 3: Rationale of the study and aims

As mentioned before, the prevalence of NPHPT in the literature varies considerably. The reasons for this variation are that different definitions and methodologies were used in the reporting studies; not all causes of secondary hyperparathyroidism were excluded, and some studies did not test the persistence of high PTH and normal calcium before evaluating the prevalence; thus the results were probably overestimated. Moreover, the prevalence has not been studied in a United Kingdom-only population in the past and the numbers may differ because of vitamin D deficiency and different genetic background.

The data on the natural history of this disease are also sparse and inconclusive. Some studies show progression to hypercalcemia, but others fail to show that. The way that many studies identify NPHPT patients, is by testing calcium and PTH at baseline, and then they retest the patients after some time to check whether they progressed to hypercalcemia. Since the persistence is not always checked at baseline, the result of subsequent hypercalcemia should be treated with caution. The variability of calcium in NPHPT during the follow-up period has been poorly described.

According to the proceedings of the Fourth International Workshop on Asymptomatic Primary Hyperparathyroidism, NPHPT remains incompletely described, especially regarding its epidemiology, natural history and management. The criteria are not clear concerning how many measurements are needed to establish a diagnosis, which makes the matter complicated for the clinician and confusing for the patient. This study aimed to identify the prevalence of NPHPT and study the natural history of this disorder in more detail. It also aimed to compare the variability of calcium between PHPT and NPHPT patients.

Normocalcaemic hypoparathyroidism could be the subclinical form of hypoparathyroidism. Patients having this disorder might be more prone to developing hypocalcaemia after they receive medications that can affect calcium levels (eg bisphosphonates, loop diuretics, denosumab). Further information on this disorder are needed before any official recommendations are made but NHYPO may have to mentioned in any future guidelines on hypoparathyroidism; its pathophysiological counterpart, normocalcaemic hyperparathyroidism, is already part of international guidelines.

The primary aims of this study were to identify the prevalence of NHYPO and to study the natural history of the disorder. We considered that NHYPO could be a consequence of greater changes in serum calcium and PTH over time, so a second aim was to compare the variability of adjusted calcium between NHYPO patients and a group of normal individuals from the same cohort.

1.3.1 Aims

1.3.1.1 Normocalcaemic hyperparathyroidism

- To study the prevalence of normocalcaemic hyperparathyroidism by using information from a referral centre in the United Kingdom
- To study the natural history of this disorder over time and identify whether there is progression to primary hyperparathyroidism
- To calculate the within-subject standard deviation of adjusted calcium in this group and compare it with the one from patients having primary hyperparathyroidism and a group of normal individuals from the same cohort

1.3.1.2 Normocalcaemic hypoparathyroidism

- To study the prevalence of normocalcaemic hypoparathyroidism by using information from a referral centre in the United Kingdom
- To study the natural history of this disorder over time and identify whether there is progression to hypocalcaemic hypoparathyroidism
- To calculate the within-subject standard deviation of adjusted calcium in this group and compare it with the one from patients having hypoparathyroidism and a group of normal individuals from the same cohort

Chapter 2: Methods and method development

Section 1: Methods

Section 2: Method development

Section 1: Methods

2.1.1 Study setting

In brief, the aims of the following studies were to study the prevalence and natural history of two newly described calcium disorders, called normocalcaemic hyperparathyroidism (NPHPT) and normocalcaemic hypoparathyroidism (NHYPO) in a metabolic bone centre referral population. In order to do that, information on patient data and several laboratory measurements, had to be identified.

Data from patients referred for a bone mineral density (BMD) measurement were retrospectively evaluated. All the patients included in the database had to have a laboratory evaluation, including calcium and parathyroid hormone, performed within a twenty-eight day period from their scan. The day of the laboratory investigations was defined to be the index day. More results of calcium and PTH before and after the index day were retrieved from hospital records and were used to study the natural history of the disease.

2.1.2 The referral centre: general information

The study was performed in the Metabolic Bone Centre in Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT). STH NHS Trust was created from merging the Central Sheffield University Hospitals and the Northern General Hospital NHS Trusts on the 1st April 2001. At the time of the study, part of the Trust were the main two adults' hospitals of Sheffield, the Northern General Hospital (NGH) and the Royal Hallamshire Hospital (RHH), as well as the more specialised ones like Jessop Wing (obstetric services), Weston Park Hospital (cancer services) and Charles Clifford Dental Hospital.

At the time of the study, the Metabolic Bone Centre (MBC) was based at the Sorby wing of the Northern General Hospital. It was one of the largest of its kind in the UK and provided care to South Yorkshire and beyond. The centre offered direct-access fracture risk assessment to both general practitioners and hospital consultants and received approximately 700 referrals per month for the fracture risk assessment service (FRAS).

Further management in the metabolic bone clinic was offered for patients with severe osteoporosis in whom initial investigations suggested an underlying metabolic bone problem. Patients with other metabolic bone diseases including Paget's disease, osteomalacia, and parathyroid bone disease were also assessed in the centre. The department was also a tertiary referral centre for less common metabolic bone disorders such as fibrous dysplasia, osteogenesis imperfecta and hypophosphataemia and there were transition clinics in collaboration with medical practitioners from the Sheffield Children's Hospital to allow smooth transition to the adult services.

The centre had an active programme of research into the pathogenesis, diagnosis, and treatment of osteoporosis and other metabolic bone diseases and it worked closely with the Academic Unit of Bone Metabolism (AUBM) of the University of Sheffield.

2.1.3 Data collection

The extraction of the data was performed by the Medical Imaging and Medical Physics department. The hospital had data from 1/12/2011 onwards. All the databases were received in Microsoft Excel Software.

Any extra information was collected using data from the patients' medical records and different systems available on NHS computers:

- Fracture risk assessment reports
- PACS and CRIS (imaging systems)
- ICE (laboratory and imaging reports). Results were available from 2009 onwards
- Lorenzo (electronic record for secondary care)
- Clinical portal (access to primary care clinical information including prescribing details in a proportion of patients)

2.1.4 Ethics

All the patients were assigned a unique study number after the extraction and any data used outside NHS computers were pseudo-anonymised.

The analysis described into the thesis did not require ethics approval; according to the Sheffield Teaching Hospitals Clinical Research Office, it fell under the "case note review" category.

2.1.5 Fracture Risk Assessment at MBC

As mentioned above, the Metabolic Bone Centre at STH, provided fracture risk assessment to patients referred from both general practitioners and hospital consultants. Reasons for referral included the following at the time of the study:

- Patient with a history of low trauma fracture
- Patient on, or commencing, systemic glucocorticoid treatment. This included patients taking steroids for more than three months or if high dose steroids was initiated (prednisolone >15mg)

- Osteopenia reported on an X-ray by a radiologist
- Patients with a disease or medication known to cause osteoporosis (malabsorption, inflammatory conditions, cystic fibrosis, endocrine disorders, use of Depo-Provera, aromatase inhibitors, androgen deprivation therapy, eating disorders with amenorrhea etc)
- Fracture probability by FRAX indicating need for BMD measurement or treatment

All patients undergoing fracture risk assessment at the MBC, had BMD measurement of the lumbar spine and proximal femur and completed a risk factor questionnaire. From August 2008 an extended one-stop assessment incorporated Vertebral Fracture Assessment (VFA) scans in patients at high risk of vertebral fracture. The indications included:

- All women over 65 years and men over 70
- Patients over 50 years but below the above age ranges with
    - Kyphosis
    - Height loss >2cm between scans or >5cm since age 25
    - Corticosteroids
- Patients in whom the lumbar spine DXA suggested a vertebral fracture
- Patients with hip fracture
- Patients with low BMD for age (Z-score < -2.0)

Spine radiographs were performed if the VFA suggested a vertebral fracture. Finally, laboratory investigations were performed to look for underlying causes of osteoporosis when clinically indicated. The indications were any of the following:

- low BMD for age (Z-score < -2.0)
- confirmed vertebral fracture

- unexplained, accelerated bone loss since the previous scan (>4.5% decrease, inappropriate for the duration of scans, any weight change and menopausal or treatment status). The expected age loss according in postmenopausal women and older men is 1%. In the first four to five years after menopause, this can increase to 2%
- if a clinical reason had been identified at the point of referral or during the BMD assessment (after discussion with clinician)

The investigations included the following:

- full blood count, erythrocyte sedimentation rate (ESR)
- bone profile [calcium, albumin, adjusted calcium, phosphate, total protein, albumin, globulin, alkaline phosphatase, creatinine, estimated glomerular filtration rate (eGFR)]
- Gamma-glutamyl transpeptidase (GGT), thyroid stimulating hormone (TSH), parathyroid hormone (PTH)
- Serum and urine electrophoresis
- Testosterone in men
- Follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin in women with premature menopause before age 40
- Anti-endomysial antibody
- 25(OH)D

All the FRAS studies were assessed and reported by specialised medical practitioners and provided recommendations regarding management, follow up and future referrals.

2.1.5.1 Bone mineral density measurement

2.1.5.1.1 Principles

Dual-energy X-ray absorptiometry (DXA) scanning has been in use since the late 1980s and it is the gold standard method for measuring bone mineral density (BMD). The scanners use X-rays, which are a type of high energy electromagnetic radiation, with a wavelength of 0.01 to 10 nm. In DXA the goal is to measure the amount of bone mineral (hydroxyapatite).

Serial DXA measurements allow the assessment of progression in BMD. However, there are several causes of inconsistency or "noise" between scans. These can be due to a number of reasons which can be patient, instrument and operator-related. Each centre performing DXA measurements should calculate the precision error which is a measurement of reproducibility. This is important when comparing BMD results over time; the change in BMD observed might be significant or simply a result of the "noise" of the test. The way to calculate this is by repeating the test in fifteen patients three times or in thirty patients twice. The patients should be similar in age, gender and bone density as the ones usually evaluated in the service, so volunteers should be avoided. The scans are usually performed on the same day after repositioning the patient. The standard deviation (SD) for each patient and the mean SD of the group are then calculated. The precision error is the root mean square standard deviation in absolute terms (g/cm^2). The least significant change (LSC) is the least amount of change in BMD that can be considered as statistically significant. The International Society for Clinical Densitometry (ISCD) recommends calculating the LSC for a 95% confidence level, which is done by multiplying the precision error by 2.77 (https://www.iscd.org).

2.1.5.1.2 DXA in the assessment of osteoporosis

DXA is mainly used to scan the spine at a anteroposterior projection (L1-L4) and the hip (axial DXA). Two sites are used to increase the diagnostic sensitivity. Peripheral DXA measurements like the non-dominant forearm can be performed in certain occasions (for example hyperparathyroidism and when hip and/or spine cannot be measured) (ISCD, 2019). The result is usually expressed in a T-score, which is the difference between the patient's BMD and the mean BMD of healthy young adults matched for gender and ethnicity, divided by the young adult population standard deviation (Blake et al, 2013).

The World Health Organization (WHO) and the International Osteoporosis Foundation (IOF) recommend that osteoporosis should be defined on the basis of BMD compared to the Third National Health and Nutrition Examination Survey (NHANES III) female reference data for all ethnicities and genders. This database has measurements for femoral neck in white females aged 20-29 years old. Osteoporosis is defined as a T-score of 2.5 SD or more below the female adult mean, osteopenia as T-score <−1.0 and >−2.5 SD and normal as a T-score greater or equal to -1.0 SD (Kanis et al, 2008).

A different way to report DXA scans is by using the Z-score, which is following a similar principle to the T-score, but in this case comparing the patient's BMD with a healthy subject matched for gender and ethnicity but also for age and dividing the difference by the age matched population SD. Z-scores are the preferred way of reporting in younger people (Blake et al, 2013).

Vertebral Fracture Assessment (VFA) is a technique performed in DXA scanners for detecting vertebral fractures. This requires only two images to capture both the thoracic and lumbar spine (posteroanterior and lateral views) compared with four images that would be

required with conventional radiography. VFA is indicated in patients of high risk of a vertebral facture (ISCD, 2019).

2.1.5.1.3 DXA scanning at the Metabolic Bone Centre

At the time of the study, there were three Hologic DXA scanners within the centre. The LSC used in the MBC was 4.5% for the spine and hip.

There were three scanning rooms in the department and each room had a different scanner. At the time of the study, these were the scanners in use:

- Room A: Hologic Discovery A in use since 2010
- Room B: Hologic QDR4500A was in use from 2011 until the 11th September 2013. The scanner was then decommissioned and replaced by a Hologic Discovery SL
- Room C: Hologic Delphi C was in use until the 3rd November 2014. The scanner was then decommissioned and replaced by a Hologic Horizon A

2.1.5.1.4 Anthropometric data

All patients coming for a DXA scan had their height (in centimetres) and weight (in kilograms) taken by the technicians (wherever possible) and this was recorded in the scan result. This information was retrieved and the body mass index (BMI) was calculated using the following equation (recorded in one decimal place)

$$BMI\ (kg/m^2) = 10000 * weight\ (kg)/ [height\ (cm)]^{2}$$

In some cases, there were extreme BMI values calculated (>100kg/m^2). For some of them, it was obvious that the height and weight had been reversely recorded in the database, and

so they were corrected automatically. For the rest, the next step was to check the form completed by the technician on the day of the BMD measurement and correct the data accordingly.

2.1.6 Laboratory measurements at Sheffield Teaching Hospitals

As mentioned above, a laboratory work-up was performed at MBC to check for underlying causes of osteoporosis when clinically indicated.

All the samples were analysed in the chemical laboratory, STH NHS FT. Blood at the MBC can be drawn at any time of the day, so patients were not necessarily fasting. Only the methods used for the measurements of the different tests described in the thesis are summarised here.

The final analysis only contains measurements performed after January 2013, as the laboratory changed the manufacturer to Roche Diagnostics (Roche Diagnostics GmbH, Mannheim, Germany) at that point and it was decided that the changes could affect the results of the thesis. However, some of the results of the intermediate analyses included measurements performed between 2011 to 2013 and so the methods are briefly described below. The information on the laboratory methods performed with Roche analysers was obtained from https://dialog1.roche.com/gb/en_gb/eLabDoc.

2.1.6.1 PTH measurement

Intact PTH (second generation) was measured throughout the whole period using an electrochemiluminescence immunoassay (“ECLIA”) by the Roche Cobas 8000 e602

analyser (Roche Diagnostics GmbH, Mannheim, Germany). The protocol number where this information was obtained from Roche's website is ms_11972103122V24.0.

Blood can be collected in a serum or EDTA plasma tube. If collected in serum, the blood should be centrifuged due to the short half-life of PTH, so EDTA is preferred as it is stable for longer (serum: stable for 8 hours at 15-25 °C, two days at 2-8 °C, six months at -20 °C; plasma: stable for two days at 15-25 °C, three days at 2-8 °C, six months at -20 °C) (Roche, 2019).

The assay we used was second-generation. The assay employs a sandwich test principle which uses two antibodies specific for different epitopes of the antigen (in this case PTH). The first antibody is coated on the surface of the well plate and is used to facilitate the immobilization of PTH. The other antibody is used to produce a measurable signal to assist with the detection of the antigen. In the case of intact PTH measurement, the N-terminal fragment (1-37) reacts with a biotinylated monoclonal antibody (mouse), while the C-terminal domain (38-84) reacts with a ruthenium complex labelled monoclonal antibody (mouse). The process starts with a first incubation using some of the sample, the biotinylated monoclonal PTH-specific antibody and the labelled PTH-specific antibody. These form a sandwich complex. During the second incubation, streptavidin-coated microparticles are added and the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The mixture is aspirated to the measuring cell where the microparticles are magnetically captured on the electrode. Any unbound substances are removed. In order to receive a measurable result, a voltage is then applied to the electrode and chemiluminescent emission is produced which can be measured by a photomultiplier (Roche, 2019).

In May 2015, the units for PTH were changed from ng/L to pmol/L, without a change in the assay. The reason for that was to be in line with the national recommendations for reporting

units and the General Practitioner medical records software stopped accepting results in ng/L. In order to convert pmol/L to ng/L, the value has to be multiplied by 9.43, while the conversion factor from ng/L to pmol/L is 0.106.

The PTH assay uses two monoclonal mouse antibodies. For people who have pet mice or work with mice, there is a risk that they will have human anti-mouse antibodies (HAMA) which can potentially interfere in the assay. The effect could be to make the result falsely high or low. This would depend on the target of the HAMA (therefore is patient-specific) and how this alters the normal binding of the reaction complex.

As the assay uses biotin-streptavidin binding, high dose biotin therapy (vitamin B7) has the potential to affect the assay. Roche quotes that samples should not be taken from patients receiving large doses (>5mg/day) for at least eight hours after they had the supplement. Most over the counter supplements have concentrations in the µg range so it is very unlikely these will have any influence on the results. Biotin causes a negative interference. This means that the patients' results will be lower than expected. If there is another source of biotin in the serum, this can bind to streptavidin in place of the biotin-labelled anti-PTH antibody.

At the time of the study, the interassay coefficient of variation (CV) measured in the laboratory was 2.2 – 3.2% at 3.6 pmol/L, 1.6 – 1.7% at 10 pmol/L and 1.4 – 1.8 at 89 pmol/L, while the reported reference range was 15-65 pg/mL (ng/L) or 1.6 - 6.9 pmol/L.

2.1.6.2 Calcium measurement

From February 2011 until January 2013, serum calcium was measured with a colourimetric assay on a Roche/Hitachi Cobas automated clinical chemistry analyser using o-

cresolphthalein complexone (o-CPC). This chemical reacts with calcium ions under alkaline conditions to form a violet coloured complex. The addition of 8-hydroxyquinoline prevents interference with magnesium and iron. The colour intensity is directly proportional to the calcium concentration and can be measured photometrically (Roche, 2019).

The laboratory introduced a new method on the 7th January 2013. The protocol number where the information was obtained is 05928222001V5.0. This method is performed on a Roche/Hitachi Cobas 8000 e702 automated clinical chemistry analyser and uses 5-nitro-5'-methyl-BAPTA (NM-BAPTA) reagent, which forms a complex with calcium ions under alkaline conditions. During a second step, the calcium ions are released by EDTA which causes a change in the absorbance of NM-BAPTA. This change in absorbance is directly proportional to calcium and can be measured photometrically (Roche, 2019).

The sample should be processed so that serum or plasma are separated from blood cells as soon as possible, as prolonged contact with clots can cause a decrease in the calcium levels. Urine samples should be collected in containers having hydrochloric acid (HCL) to prevent calcium salt precipitation. The reported stability in serum/plasma is seven days at 15-25 °C, three weeks at 2-8 °C and eight months at -15 to -25 °C. In urine, the stability is two days at 15-25 °C, four days at 2-8 °C and three weeks at -15 to -25 °C. No significant interference is reported with icterus, haemolysis, lipemia, magnesium and common therapeutic concentrations of drugs. In rare cases, Waldenström's macroglobulinemia, may cause unreliable results in serum measurements (Roche, 2019).

The interassay coefficient of variation as measured in the laboratory is 1.1 – 1.5% at 1.52 mmol/L and 0.6 – 1.1% at 3.07 mmol/L.

2.1.6.3 Albumin measurement

Albumin displays a sufficient cationic character at a pH of 4.1 and binds with bromcresol green (BCG), an anionic dye, to form a blue-green complex. The colour intensity is directly proportional to the albumin concentration in the sample and can be measured photometrically. The measurement was performed using a Roche/Hitachi Cobas 8000 e702 analyser. This colourimetric method was introduced in 2011 and has not changed since then. The protocol number where the information was obtained is 0005166861190c701V6.0. No significant interference is reported with icterus, haemolysis, lipemia, magnesium and common therapeutic concentrations of drugs. In rare cases, Waldenström's macroglobulinemia, may cause unreliable results in the measurements (Roche, 2019).

The interassay coefficient of variation as measured by the laboratory is 1.5 – 2.4% at 33.9 g/L and 1.0 – 1.7% at 59.7 g/L.

2.1.6.4 Albumin-adjusted calcium calculation

Total calcium can vary significantly depending on protein concentration as approximately forty percent is protein bound, mainly to albumin. Adjusting total calcium for albumin concentration is therefore a more practical means of determining serum calcium (Barth et al, 1996). The equations used to calculate that in the laboratory since 2011 are shown in Table 2-1. The Pathology Harmony reference range (Berg, 2012) has been in use for adjusted calcium since 2011; the range reported is 2.20 - 2.60 mmol/L. The multiplication factor to convert to mg/dl is 4.008. This is the reference range that is used for this study.

	Time introduced	Equation for albumin adjusted calcium	Comments
1	February 2011	= Total calcium + [0.02 (41.5-albumin)]	Introduced after the change in methods of calcium and albumin as a temporary measure until the laboratory would establish its own equation
2	15 November 2011	= Total calcium + [0.0184 (45.7-albumin)]	The laboratory used accumulated local data to calculate a new equation. The average increase in adjusted calcium was 0.08 mmol/L
3	20 February 2012	= Total calcium + [0.02 (41.5-albumin)]	Due to the increase in mean adjusted calcium with the previous equation, concerns were raised about inappropriate numbers of patients with an elevated adjusted calcium and the effect this was having on patient care. Therefore, the laboratory decided to revert back to the original equation
4	11 September 2014	= Total calcium + [0.0172 (43-albumin)]	The new method for calcium measurement was introduced on the 14th January 2013 and the new equation was introduced a few months later. This equation gave an average increase in adjusted calcium of 0.03 mmol/L

Table 2-1: Equations used by the chemical laboratory in Sheffield Teaching Hospitals after 2011 to calculate albumin adjusted calcium

2.1.6.4.1 Method of establishing the final adjusted calcium equation in the laboratory

The method that the laboratory used to calculate this equation is described below. Information was retrieved from the laboratory's Standard Operating Procedures (SOP), obtained from the Clinical Chemistry Department, STH NHS FT. The method is based on the paper by Barth et al (Barth et al, 1996).

2.1.6.4.1.1 SOP (Procedure No. CCCLIN002, revision 2, date 14/1/16)

1. Request I.T. department to perform a search for all NGH and RHH total calcium results between the required period, (e.g. three months), which fit the following criteria: patient ≥ 18 years old, albumin (ALB) ≤ 55 g/L, urea (UR) ≤ 15 mmol/L, creatinine (CR) ≤ 200 µmol/L, potassium (K) ≥ 3.5 mmol/L, AST (AST) ≤ 40 U/L and/or ALT ≤ 41 U/L, alkaline phosphatase (ALP) ≤ 130 U/L. The search should be performed after excluding the locations: Endocrinology, Haematology, Oncology, Nephrology/Renal, General surgery. Only one total calcium and paired albumin result per patient should be included
2. Manually exclude data points with an albumin concentration less than 20 g/L and for females only those with an AST of ≥ 33 U/L and/or an ALT of ≥ 34 U/L
3. Using Microsoft Excel plot a graph of total calcium (y axis) against albumin (x axis)
4. Create a trendline using least squares regression. The regression equation of y = mx + c should then be determined
5. Substitute total calcium and albumin terms into the regression equation and manipulate as outlined in the example below:
    a. Regression equation y = 0.0172x + 1.58 becomes Total calcium (TCa) = 0.0172[Albumin] + 1.58
    b. Adjusted calcium (ACa) = Total calcium (TCa) – 0.0172[Alb] + (mean total calcium – 1.58)
    c. If the total calcium reference range is 2.20 – 2.60 mmol/L the mean calcium of reference range is 2.40, then 2.40 – 1.58 = 0.82
    d. ACa = TCa – 0.0172 ([Alb] – 0.82/0.0172)
    e. ACa = TCa – 0.0172 ([Alb] – 47.7)
    f. Re-arranging into the usual format gives: ACa = TCa + 0.0172 (47.7 – [Alb]).

6. Check the validity of the new equation by entering the old and new versions into a spreadsheet containing the total calcium and albumin results from the search and calculating the adjusted calcium concentration achieved with both equations
7. Limitations: The relationship between calcium and albumin is not the same across the range of possible albumin concentrations often resulting in a different slope and therefore adjustment equation at the lower and higher ends of the range. However it has been shown that the difference in adjusted calcium results obtained if two separate equations are used (as opposed to one over the entire range) is small and not clinically significant (Barth et al, 1996). The equation is not reliable in patients with significant dysproteinaemias, acid-base disturbances or albumin concentrations <20 g/L. In critically ill patients abnormal calcium binding may lead to a falsely normal or high adjusted calcium result. The equation may not be appropriate in paediatric patients less than eighteen years old.

2.1.6.4.1.1.1 Results (obtained from laboratory, as per document May 2013)

In order to calculate the equation, the laboratory had to collect enough data. The search retrieved a total number of data points of 10431 patients (3316 GPs + 7115 Hospital). Initially all the data points were used to create the new equation. The mean total calcium value used was 2.40 mmol/L, being the mean of the Pathology Harmony reference. When compared to the already existing equation until 2014, ACa = TCa + 0.02 (41.5 – [Alb]), this gave a mean increase in adjusted calcium of 0.11 mmol/L. As this seemed to be a particularly high increase other possibilities (

Table 2-2) were tried to see if any made a difference.

Combinations tried:

1. GP patient samples only

2. Hospital patient samples only
3. Data points included only if the albumin concentration had >100 calcium results in total. This was done because in the Barth et al paper (Barth et al, 1996) they ensured they had >100 total calcium results for all their albumin concentrations. For this data it was only possible for albumin concentrations of 32 to 51 g/L, but this does cover the reference range
4. Mean total calcium of the data set was used instead of mean of the reference range i.e. the mean total calcium for all 10 431 was 2.32 mmol/L. The same was done for the data set where the albumin concentrations had >100 total calcium results

Data set	**n**	**Mean total calcium used**	**Albumin range**	**ACa equation**	**Mean increase in ACa**	**Range of difference in ACa**
All	10431	2.40	20 to 55	TCa + 0.0172 (47.7 - [Alb])	0.11	0.05 to 0.14
GP	3316	2.40	26 to 55	TCa + 0.0142 (47.9 - [Alb])	0.11	0.00 to 0.17
Hospital	7115	2.40	20 to 55	TCa + 0.0177 (46.9 - [Alb])	0.10	0.05 to 0.13
TCa n >100 for each alb.	9734	2.40	32 to 51	TCa + 0.0165 (47.3 - [Alb])	0.10	0.06 to 0.13
All	10431	2.32	20 to 55	TCa + 0.0172 (43 - [Alb])	0.03	-0.03 to 0.06
TCa n >100 for each alb.	9734	2.33	32 to 51	TCa + 0.0165 (43 - [Alb])	0.03	-0.01 to 0.06

Table 2-2: Summary of all the data set combinations tried for the adjusted calcium equation and their results

The data in Table 2-2 shows that of all the various combinations tried, the only factor which made a notable difference to the average increase in adjusted calcium was using the mean total calcium of the data set rather than the mean of the Pathology Harmony reference range. Although this is not in line with Pathology Harmony, a rise of this level would fit with

improving the slightly negative bias that the laboratory was running on quality assessments. Using the data set mean of 2.32 mmol/L to derive the equation could be somewhat justified by the fact that it is also the mean of the recommended Roche reference range.

Using a mean adjusted calcium of 2.32 mmol/L and the Pathology Harmony reference range, this would result in 8.8% of the population being outside the reference range. According to the Clinical & Laboratory Standards Institute (CLSI), this percentage should be kept under 10%.

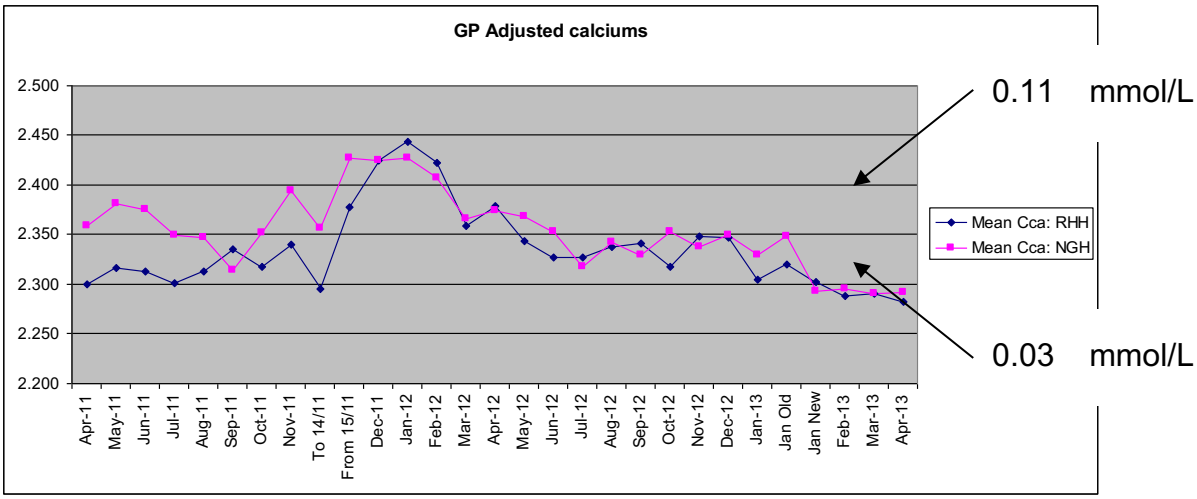


Figure 2-1: Monthly GP adjusted calcium data over 2011 to 2013 in Sheffield Teaching Hospitals.

The arrows on show the likely increase in GP average adjusted calcium concentration with an increase of 0.11 and 0.03 mmol/L respectively. Cca: corrected calcium; RHH: Royal Hallamshire Hospital; NGH: Northern General Hospital

2.1.6.5 25(OH)D measurement

25(OH)D was measured using the IDS-iSYS automated immunoassays (Immunodiagnostic Systems, Boldon, UK) until 2013. This method uses a specific 25(OH)D antibody which is labelled with acridinium following a pre-treatment stage to release vitamin D binding protein.

Magnetic particles that link to 25(OH)D are added and these are attracted by a magnet. Following that, trigger reagents are added and light is emitted by the acridinium, which is inversely proportional to the concentration of 25(OH)D. The protocol number where this information was obtained is IS-2700PLV05 (UK-Immunodiagnostic-Systems, 2011).

The method changed from an immunoassay to an electrochemiluminescence binding assay in 2013, performed by Roche modular analytics Cobas E170 (Roche Diagnostics GmbH, Mannheim, Germany). The protocol number where the information was obtained is ms_05894913190V7.0. This method uses recombinant vitamin D binding protein (VDBP) rather than antibodies. This is able to bind vitamin D2 and D3. The process involves incubating the sample with dithiothreitol and sodium hydroxide, so that vitamin D can be released from the VDBP. Then, the sample is incubated with ruthenium labelled vitamin D binding protein and a complex between the vitamin D and the ruthenylated vitamin D binding protein is formed. Streptavidin-coated microparticles and vitamin D labelled with biotin, are then added to the sample. A complex consisting of the unbound ruthenylated vitamin D binding protein and the biotinylated vitamin D is formed and becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed. Application of a voltage to the electrode then induces chemiluminescent emission which can be measured by a photomultiplier (Roche, 2019).

The laboratory interassay coefficient of variation for this assay is 6.5 – 9.9% at 48.2 nmol/L and 4.5 – 6.3% at 92.3 nmol/L. The 25(OH)D units used in this report are nmol/L. A multiplication by 0.4 is needed to convert the units to ng/ml (or multiply by 2.5 to convert ng/ml to nmol/L).

2.1.6.6 Creatinine measurement

Creatinine was measured using a Roche/Hitachi Cobas c8000 e702 analyser (Roche Diagnostics GmbH, Mannheim, Germany). The protocol number where the information was obtained is 0006407137190c701V11.0. This automated assay is based on the Jaffé method; creatinine forms a yellow-orange complex with picric acid in an alkaline solution and the dye formation is proportional to the creatinine concentration in the specimen (Roche, 2019).

The interassay coefficient of variation for this assay in the laboratory was 2.7 – 6.1% at 55.7 µmol/L and 2.3 – 4.0% at 458 µmol/L.

According the Kidney Disease Improving Global Outcomes (KDIGO) guidelines, serum creatinine should be measured using assays traceable to pure creatinine standards via a valid calibration hierarchy. These should be specific and minimally-biased compared with isotope-dilution mass spectrometry (IDMS) reference method results. Results should be traceable to reference materials and methods listed on the Joint Committee for Traceability in Laboratory Medicine (JCTLM) database (KDIGO, 2012). The Jaffe assay used by STH laboratory was standardized against IDMS (Roche, 2019).

2.1.6.7 Glomerular Filtration Rate (GFR) and Chronic Kidney Disease (CKD) stages

Chronic Kidney Disease stages are classified using the level of Glomerular Filtration Rate (GFR) as there is a link established between its level and adverse outcomes including mortality (Table 2-3). In the absence of evidence of kidney damage, categories G1 and G2 do not fulfil the criteria for CKD (KDIGO, 2012).

Category of GFR	GFR (ml/min/1.73 m^2)	Term used
G1	≥90	Normal or high
G2	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	<15	Kidney failure

Table 2-3: Chronic kidney disease stages according to glomerular filtration rate (GFR). Adapted from the original (KDIGO, 2012)

Several equations have been developed throughout the years for the calculation of GFR and creatinine clearance (CrCl) in adults. In summary, the equations using creatinine need to include age, sex, race and body size to account for creatinine production by the muscles. The equations described below use serum creatinine (SCr) in mg/dL. To convert creatine into different units, 1 mg/dL = 0.01131222 μmol/L or 1 μmol/L = 88.4 mg/dL.

The first equation was the Cockcroft-Gault published in 1976. This was

$$CrCl\left(\frac{mL}{min}\right) = (140 - age)\, x \frac{weight}{72 \times SCr}\, x\, 0.85\ (if\ female)$$

The problem with equations like the Cockcroft-Gault is that they were developed before the standardisation of creatinine assays. Further equations after the standardisation of the assay were then established (KDIGO, 2012). The six-variable (age, sex, ethnicity, serum creatinine, urea, and albumin) was followed by the four-variable Modification of Diet in Renal Disease (MDRD). Study equations were developed and used (Levey et al, 1999; Levey et al, 2006). The four-variable equation can be seen below:

$$eGFR\left(\frac{mL}{min \times 1.73\ m^2}\right) = 175 \times (\text{Serum creatine})^{-1154} \times age^{-0.203} \times [0.742\ if\ female] \times [1.212\ if\ black]$$

Finally, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was developed in 2009. In order to develop this equation, data from 8254 participants from six research studies were evaluated.

$$eGFR\left(\frac{mL}{min \times 1.73\ m^2}\right) = 141 \times \min\left(\frac{Scr}{k}, 1\right)^{\alpha} \times \max\left(\frac{Scr}{k}, 1\right)^{-1.209} \times 0.993^{age} \times 1.018\ [if\ female] \times 1.159\ [if\ black]$$

Scr is serum creatinine in mg/dL, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, max indicates the maximum of Scr/κ or 1 (KDIGO, 2012).

When the later equation was validated, it performed better than the MDRD Study equation especially at higher GFR levels (GFR ≥60 ml/min/1.73 m^2). There was lesser bias (median difference between measured and estimated GFR), improved precision and greater accuracy (Levey et al, 2009). Due to the fact that GFR matches physiologically to kidney size, which is related to the body surface area, the interpretation of GFR is based on comparison to normative values adjusted for body surface area. The value 1.73m^2 used in the units, is the average value of the body surface area (BSA) in 25-year old adults in the USA in 1927. This value was maintained throughout the years for normalisation purposes (KDIGO, 2012).

The STH Clinical Chemistry Department changed the used equation for estimating eGFR from the MRDR to the CKD-EPI in August 2015.

2.1.6.8 Alkaline phosphatase (ALP) measurement

Alkaline phosphatase was measured using a Roche/Hitachi Cobas c8000 e702 analyser. The protocol number where the information was obtained is 0005166888190c701V8.0. The sample is added to p-nitrophenyl phosphate, which, in the presence of magnesium and zinc

ions, is cleaved by phosphatases into phosphate and p-nitrophenol. The p-nitrophenol released is directly proportional to the catalytic ALP activity and is determined by measuring the increase in absorbance. The interassay coefficient of variation for this assay was 2.4% at 92.8 IU/L and 1.7% at 224 IU/L (Roche, 2019).

2.1.6.9 Phosphate measurement

Phosphate was measured using a Roche/Hitachi Cobas c8000 e702 analyser. The protocol number where the information was obtained is 0005171377190c701V11.0. Inorganic phosphate forms an ammonium phosphomolybdate complex with ammonium molybdate in the presence of sulfuric acid. The concentration of phosphomolybdate formed is directly proportional to the inorganic phosphate concentration and is measured photometrically. The interassay coefficient was 1.4% at 1.23 mmol/L and 1.2% at 2.04 mmol/L (Roche, 2019).

2.1.7 Statistical analyses

2.1.7.1 Mahalanobis distance analysis

Since calcium and PTH are not independent variables, it was considered best to use a bivariate statistical approach to define the different categories of patients. This approach has been used in the past to study the prevalence of NHYPO (Palermo et al, 2015). The approach is called Mahalanobis distance and it is a classical way to define outliers in a multivariate space (in this case, a space with two variables, adjusted calcium and PTH).

In univariate space, calculating the distance from one point to another is straightforward and is called Euclidean distance. For example, in statistics, any point can be expressed as a distance of a number of standard deviations (SD) from the mean (standard Normal deviate).

This is calculated, using the formula (x – μ) / SD, where x is the point studied, μ is the mean and SD the standard deviation of the distribution. From that, the probability of a point belonging to the set can be calculated. For example, if a point is more than two standard deviations from the mean, is it defined as an outlier (outside the 95% of the distribution) (Altman, 1991).

Euclidean distance can also be used in multivariate space if the variables are not correlated. In this case, the points are distributed in a spherical way around the centre and the axes from the mean to two different points are in an angle of 90^0 from each other. However, when the variables are correlated, calculating the distance becomes more complicated (Figure 2-2). In this case, the probability of a point belonging to the dataset depends not only from the distance, but also from the direction (Wikipedia, 2017). Hence, the shape of the cluster of points has to be considered.

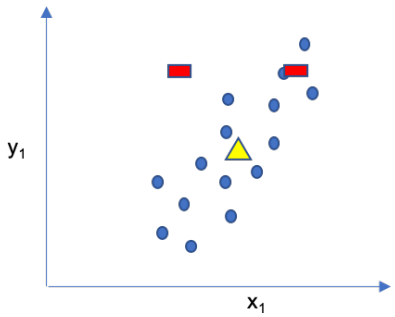


Figure 2-2: Example showing that when two variables are correlated, the distance of two points from the mean cannot really be calculated using Euclidean distance.

The two squares have similar Euclidean distance from the mean of the cluster (triangle) but the square on the left seems more of an outlier. Figure adjusted from original (Clapham, 2016)

Covariance is a measure of how two variables change together. In the case of the variables x_1 and y_1 in Figure 2-2, the covariance is positive; as x_1 increases, y_1 tends to increase. In

the case of multivariate data, covariance is expressed as the variance-covariance matrix. The variance covariance matrix is square, which means the number of rows and columns is the same and it contains the covariances of all pairs of variables. In the analyses described below, the number of variables analysed for each patient were two (adjusted calcium and PTH) and so the sample covariance matrix was a two-by-two matrix. In this matrix, the diagonal terms give the variances of the variables (or the covariance of a variable with itself) and the off-diagonal terms give the covariances between the variables (Figure 2-3). Covariance of (x_1 x_2) equals the covariance of (x_2 x_1), so the matrix is also symmetrical (Clapham, 2016).

$$\begin{array}{c} \\ x_1 \\ x_2 \end{array} \begin{array}{c} \begin{array}{cc} x_1 & x_2 \end{array} \\ \begin{pmatrix} \text{covar}(x_1\,x_1) & \text{covar}(x_1\,x_2) \\ \text{covar}(x_2\,x_1) & \text{covar}(x_2\,x_2) \end{pmatrix} \end{array} = \begin{array}{c} \\ x_1 \\ x_2 \end{array} \begin{array}{c} \begin{array}{cc} x_1 & x_2 \end{array} \\ \begin{pmatrix} \text{var}(x_1) & \text{covar}(x_1\,x_2) \\ \text{covar}(x_2\,x_1) & \text{var}(x_2) \end{pmatrix} \end{array}$$

Figure 2-3: A two-by-two matrix covariance matrix.

In this matrix, the diagonal terms give the variances of the variables (or the covariance of a variable with itself) and the off-diagonal terms give the covariances between the variances. Figure adjusted from original (Clapham, 2016)

Mahalanobis distance (MD), was introduced in 1936 by P.C. Mahalanobis. It is a multi-dimensional generalization of the idea of measuring how many SD away an observation is from the mean of a distribution. Mahalanobis distance creates two new axes at the cluster of points which are perpendicular to each other (Figure 2-4). MD then re-scales the two new axes in order for them to have a variance equal to one. This removes the covariance

between the variables. After doing so, the Mahalanobis distance equals the Euclidean distance (Wikipedia, 2017).

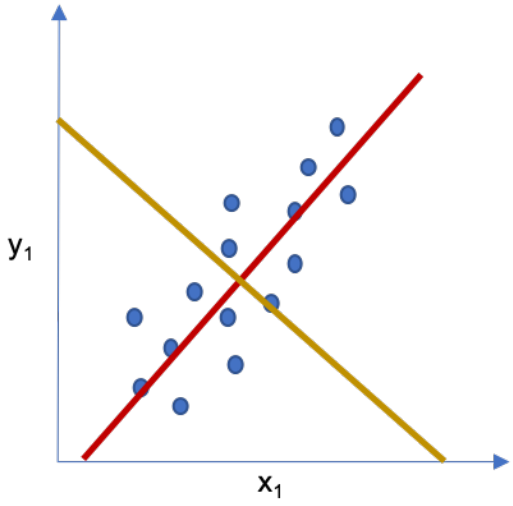


Figure 2-4: Mahalanobis distance creates two new axes in the cluster of points and one is perpendicular to the other.

The red axes, is considered to be the principal one, as most points are located along its direction. Figure adjusted from original (Clapham, 2016)

A vector in mathematics is a quantity determining the position of a point in space and it gives both direction and magnitude (Figure 2-5).

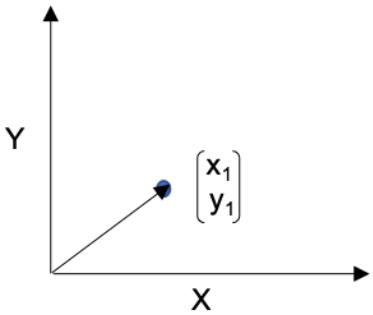


Figure 2-5: A vector is a quantity determining the position of a point in space.

The length of this vector is the squared root of $[(x_1)^2 + (y_1)^2]$. For example, if $x_1 = 4$ and $y_1 = 3$, then the length would be the square root of 25, which is 5

An eigenvector is a column vector that when multiplied with a matrix, it results in the same eigenvector multiplied by an integer called eigenvalue. The equation for this relationship is M*V= λ*V. In this case, M is a matrix, V is the column vector and λ is the eigenvalue.

The eigenvector is the principal axis of the graph at Figure 2-4. This is the direction of the greatest variability. The second eigenvector is perpendicular to the first one and they both have different eigenvalues. To remove the covariance, the axes have to be decreased by the square root of their eigenvalue ($\sqrt{\lambda}$) (Clapham, 2016)

If a matrix is a two-by-two matrix, it would have two eigenvectors. Eigenvectors are scaled to have a length of one. To do so, in the example of Figure 2-5, the scaled eigenvector would be

$$\begin{pmatrix} 4/\sqrt{25} \\ 3/\sqrt{25} \end{pmatrix} = \begin{pmatrix} 4/5 \\ 3/5 \end{pmatrix}$$

MD is defined for each point x_i, by the following equation

$$MD^2 = (x_i - \mu)^T S^{-1} (x_i - \mu)$$

MD: Mahalanobis distance, μ: arithmetic mean of the dataset and S: sample covariance matrix (as defined above). As x_i and μ are both vectors, $(x_i - \mu)$ is a matrix. T indicates that the matrix has to be transposed into a new matrix (where the rows of it are the columns of the first one).

MD is a classical method of defining outliers in a multivariate point cloud. It is known that if the data follow a two dimensional Gaussian distribution, then the squared Mahalanobis distances would approximately follow a chi-squared distribution with two degrees of freedom (Van Aelst & Rousseeuw, 2009). In the analysis described below, the observations were

considered outliers if $MD^2 > X^2_{2;\ 0.975} = 7.378$ ($97{,}5^{th}$ percentile of a chi-squared distribution with two degrees of freedom). Using this method, subjects were identified as "normal" if they were inside the ellipse and "abnormal" if they were outside the ellipse.

2.1.7.2 Calculation of the within-subject standard deviation

As mentioned above, the day of the laboratory investigations was defined to be the index day. More results of calcium and PTH before and after the index day were retrieved from hospital records and were used to study the natural history of the diseases described.

When having multiple measurements from an individual, these measurements are generally not equal to each other and tend to vary around a "true average". Each individual then has a standard deviation of these measurements which can easily be calculated. If we assume that each individual has similar standard deviation to the rest, we can then calculate the mean within-subject standard deviation (S_w). The most common way to do this, would be to average the variances of all the patients and then take the square root (Bland & Altman, 1996a). However, this method can be applied in cases where the number of measurements is the same for each subject, which is not usually the case when dealing with real-life data.

For this reason, the analysis of variance method was used to calculate the within-subject variance, as this method deals with the case of subjects having different numbers of observations. The value of the residual mean square is the within-subject variance. To follow this method, one first needs to check the assumption that the standard deviation is unrelated to the magnitude of the measurement. This can be done graphically, by plotting the individual subjects' standard deviations against their means and analytically by calculating a rank correlation coefficient (Bland & Altman, 1996a).

In order to perform this analysis, the assumption that standard deviations across the subjects are similar should be made, as described above. However, this is not true in all clinical situations, for example the standard deviation could be increased in patients that were in intensive care. Using a single "mean" within-standard deviation might underestimate the variability for some patients (Masse, 1997). We understand that this is a limitation of this approach and that the true within-subject SD might be bigger.

After calculating the within-subject SD (S_w), there are two ways of finding the confidence intervals for this value. One is done through calculating the standard error of the within-subject-SD. The standard error of s_w is given by: $SE(s_w) = \frac{s_w}{\sqrt{2d}}$, where d is the degrees of freedom, $d = \sum_{i=1}^{k}(n_i - 1)$, n is the number of measurements for each subject and k is the number of subjects. If d is greater than 30 then, the 95% confidence interval (CI) limits for s_w are then given by: $s_w \pm 1.96 \times SE(s_w)$. To use this approach, one needs to assume that within the subject, the distribution of observations is normal. This approach can be used in the case where each subject has the same number of measurements (Bland, 2011). As this is not the case in real-life data, a different approach was followed.

For a Normal population, a sample variance follows a Chi-squared distribution multiplied by the population variance and divided by the degrees of freedom. In order to follow this approach, one needs to find the 2.5% and 97.5% points of the Chi-squared distribution with the degrees of freedom for the estimate. Then, these numbers need to be multiplied by the sample variance and divide by the degrees of freedom. This gives the 95% CI for the variance. When these are squared, the calculation results to the CI for the standard deviation (Bland, 2011). This is the approach followed in this project.

2.1.7.3 Mixed linear model

For the comparison of the overall measurements of PTH and adjusted calcium resulting from follow up, I used a mixed linear model. This is a statistical model containing both fixed effects and random effects. These models are used in settings where there are repeated measurements and there is non-independence in the data. An example given in the literature is the one of observations on different patients performed by different doctors. There are different levels when studying this setting. First, is the variability of the outcome within patients seen by the same doctor. The measurements in this case are similar and are not independent. The second, is the variability of the outcome between doctors. In this case, the samples in the doctors level are independent. In order to deal with this data, one way is to aggregate the measurements from each doctor and take their average. The aggregated data would then be independent. By comparing the mean of means, one does not really take into account all the data. Another way is to analyse data from each doctor at a time. Again, this approach does not really take into account all the data. The linear mixed model, allows the utilisation of all the data and allows for correlations between the data (UCLA).

In the case of repeated measurements, each patient might have a different mean from the group and the measurements are correlated. In this project, the aim was to calculate the overall mean for adjusted calcium for each category. The linear mixed model uses fixed and random effects to do this. The fixed effects is used when comparing means of groups representing different diagnoses, treatments, groups etc. The random effects is used when the values are a sample of a larger population and they vary (Bland, 2015). Therefore, in this project, individual variability was modelled using a random effect (repeated measurements within subject) and the difference between groups (i.e. different category) was estimated using a fixed effect.

2.1.7.4 Other analyses

When one measurement was available per patient (for example to compare baseline data), appropriate tests were performed to test the hypothesis that the means between the groups are similar. Histograms were used in order to check whether the data were normally distributed. If the distribution was not Normal, the data were log transformed and checked again through histograms. In the case of normally distributed data, parametric tests were used for comparisons (Independent Student's t test for two groups and ANOVA for more than two). In the case of more than two groups, Bonferroni's correction was used for pairwise comparisons. If neither of these distributions was Normal, appropriate non-parametric tests were used (Mann-Whitney for two groups and Kruskal-Wallis for more than two groups). The chi-square test was used for the analysis of categorical data. The results are presented as mean and standard deviation when the data were normally distributed, as geometric mean and 95% confidence intervals when log transformation was performed in order to achieve a normal distribution of the data, and median and interquartile range (IQR) in the case where non parametric tests were used for the analysis. A p value smaller than 0.05 was considered as significant (Altman, 1991).

2.1.7.5 Software used for analyses

The statistical analyses have been performed using the R studio statistical software (RStudio, Inc. Boston), SPSS Statistics (International Business Machines Corporation, New York) and Microsoft Excel (Microsoft Office).

2.1.8 Definitions

Using the laboratory reference intervals for serum albumin-adjusted calcium and PTH, these patients were divided into ten different categories as described in Table 2-4. “High” or “low” are referred to values of either adjusted calcium or PTH being above or below the reference range respectively.

1. Normal	Anyone inside the ellipse
2. Hyperparathyroid hypercalcaemia (HH) Included primary hyperparathyroidism (PHPT) and familiar hypocalciuric hypercalcaemia (FHH)	Anyone outside the ellipse having high adjusted calcium and high PTH
3. Hypoparathyroidism	Anyone outside the ellipse with low adjusted calcium and low PTH
4. Secondary hyperparathyroidism	Anyone outside the ellipse with • low adjusted calcium and high PTH OR • normal adjusted calcium and high PTH with 25(OH)D<50nmol/L and/or eGFR<60 ml/min/1.73m^2
5. Non-PTH hypercalcaemia	Anyone outside the ellipse with high adjusted calcium and low PTH
6. Likely normocalcaemic hyperparathyroidism (NPHPT)	Anyone outside the ellipse having normal adjusted calcium and high PTH, given that 25(OH)D≥50nmol/L and eGFR≥60 ml/min/1.73m^2
7. Normocalcaemic hypoparathyroidism (NHYPO)	Anyone outside the ellipse with normal adjusted calcium and low PTH
8. Normoparathyroid hypercalcemia	Anyone outside the ellipse with high adjusted calcium and normal PTH
9. Normoparathyroid hypocalcaemia	Anyone outside the ellipse with low adjusted calcium and normal PTH
10. Unclassified abnormal	Anyone with normal adjusted calcium and normal PTH, but outside the ellipse

Table 2-4: Patients' categories definitions

The classification into groups was done differently in the paper by Palermo et al. After applying Mahalanobis distance, they used the geometric mean of the healthy reference range for PTH and the reference range for adjusted calcium to define their groups (Palermo et al, 2015). We have adjusted this method and brought it closer to the everyday clinical practice, by using the clinical reference ranges for both adjusted calcium and PTH.

After dividing the patients into different groups, a careful review of the medical notes of the "likely NHPER patients" was performed, to further check for other exclusion criteria as mentioned in the introduction:

- lack of persistently elevated levels of PTH on at least two consecutive measurements
- use of medications on the index day known to affect PTH levels (diuretics, lithium, denosumab, bisphosphonates)
- renal calcium loss (hypercalciuria)
- diseases of the gastrointestinal tract known to affect calcium absorption (coeliac disease, inflammatory bowel disease, bariatric surgery)
- other diseases of calcium metabolism

For the NHYPO group, the patients' medical notes were reviewed to look for other causes of the abnormalities found. Any unconfirmed data were excluded.

In terms of vitamin D levels, "low" was defined as any level lower that 50 nmol/L and "normal", a level ≥50 nmol/L. "Normal" eGFR was a level ≥60 ml/min/1.73m^2 and "low" when the level was <60 ml/min/1.73m^2. Hypercalciuria was defined as a 24-hour calcium urine level >10 mmol/24h. This was true for the first few analyses. However, this was then adapted to the international definition of 24-hour urine calcium ≥6.25 mmol/24h for women or 7.5 mmol/24h for men and/or ≥ 0.1 mmol/kg/24h (Worcester & Coe, 2008).

Section 2: Method development

2.2.1 Results from the initial analyses

There were several analyses performed before the final one due to the fact that certain problems were identified after completing each analysis. The initial results are described below. Any other intermediate analyses can be found in the Appendix along with the problems identified and the way they were resolved. Moreover, the final codes used in R studio and their explanations can also be found in the Supplementary material.

2.2.1.1 Analysis 1 (completed in 2016)

2.2.1.1.1 The database

The original database received, "SearchServiceResultsSTH15691" contained information on 9224 patients (74% female, mean age 66 years) that had a BMD measurement and a laboratory evaluation between December 2011 and May 2015. The laboratory work-up had to be performed within 28 days from the scan. The given data on adjusted calcium were used for this analysis, as calculated by the laboratory.

Information available in this database was: Age, Gender, Hospital number, T-score spine total, Z-score spine total, T-score right hip total, Z-score right hip total, adjusted calcium calculated by the laboratory, vitamin D, eGFR, PTH, PINP, 24-hour urine calcium.

2.2.1.1.2 Statistical analysis

The processing of the database was performed using the filters and several other functions in Microsoft Excel. The graphs of the natural history were also done using the same software. The Mahalanobis distance analysis was performed using the R software and was

completed by the School of Health and Related Research (ScHARR) in the University of Sheffield.

2.2.1.1.3 Baseline characteristics of the population

In order to define the patient categories according to their calcium metabolism disorders, data from only 5977 patients having both serum-albumin adjusted calcium and PTH available, were used. Out of them, 4380 (73.3%) were women. Their mean age was 66 years (range 17-100). Out of the 5479 patients that had a 25(OH)D measurement available, 45% had vitamin D deficiency (<50 nmol/L).

On the original database, there were some patients with extreme BMIs (>100kg/m^2, n=71). For some of them, it was obvious that the height and weight had been reversely recorded in the database, and so they were corrected automatically. There were four patients left with BMI>100kg/m^2. The next step was to check the form completed by the technician on the day of the BMD measurement. In order to make sure that there were no other false recordings, the ones having BMIs <14 or >50 kg/m^2 were also checked using the form completed on the day of the BMD measurement. All these recordings were confirmed.

After applying the statistical method described above, the ellipse seen in Figure 2-6 was formed.

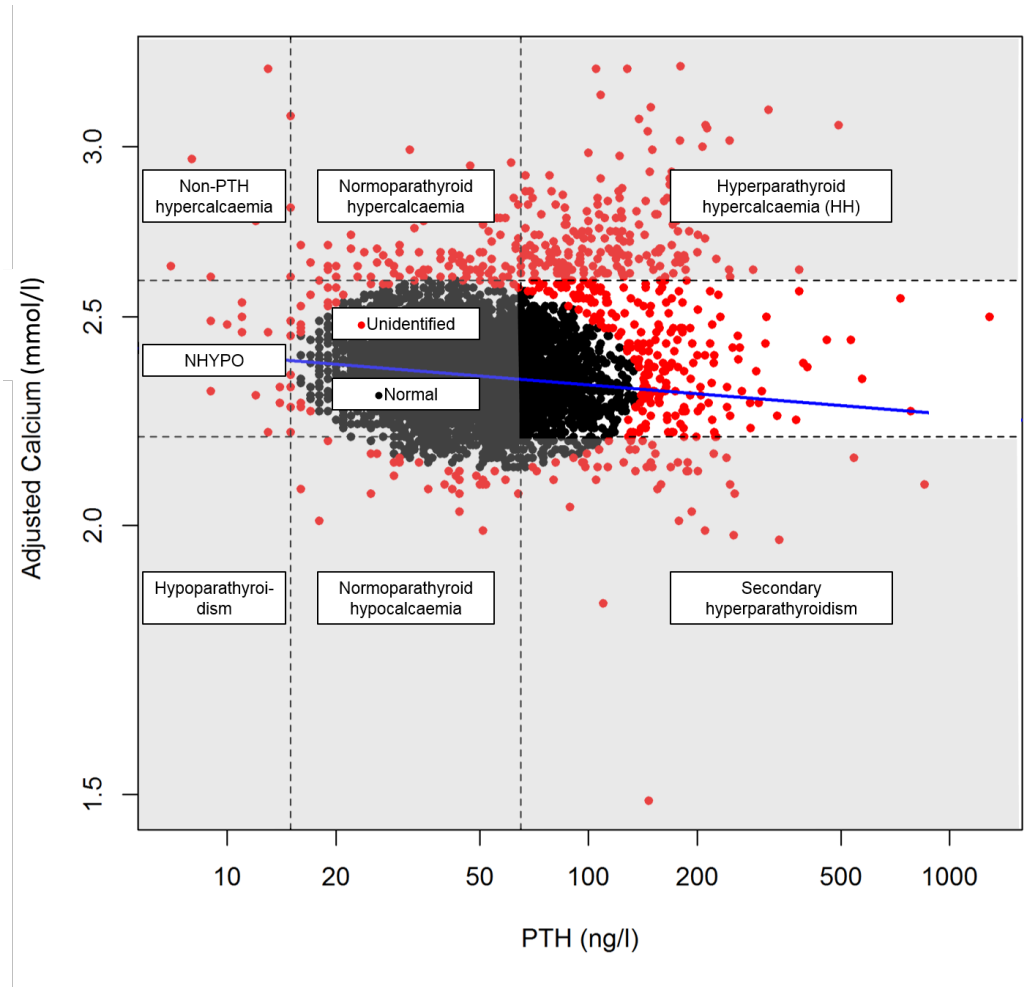


Figure 2-6: Data results from adjusted calcium and PTH from analysis 1.

The ellipse was formed using a statistical method (Mahalanobis distance) to identify "normal" subjects (black dots) and "abnormal" ones (red dots). The reference range of both adjusted calcium and PTH (horizontal and vertical dashed lines respectively), were used to identify patient categories as seen above. The white area includes patients with normal adjusted calcium and high PTH (n=235); these were either potentially NPHPT patients [given that 25(OH)D≥50nmol/L and eGFR≥60 ml/min/1.73m^2] or secondary hyperparathyroidism patients [given that 25(OH)D<50nmol/L and/or eGFR<60 ml/min/1.73m^2]. The Pearson correlation coefficient was -0.146 (95% CI: -0.172 to -0.119, P<0.001). The regression line is indicated in blue. NHYPO: Normocalcaemic hypoparathyroidism

2.2.1.1.4 Normocalcaemic hyperparathyroidism patients

Based on laboratory results on the index day, 235 patients were identified to be outside the ellipse and have normal adjusted calcium and high PTH. Using the international criteria on NPHPT, 134 patients were excluded because of either low eGFR (<60 ml/min/1.73m2) or no eGFR measurement. Moreover, 82 patients with normal eGFR, were excluded because of 25(OH)D<50nmol/L (n=71) or no vitamin D measurement (n=11). This resulted in identifying 19 patients, all women, with likely NPHPT (Table 2-5).

Categories	**Frequency**	**Percent**
Normal	5334	89.2
Hyperparathyroid hypercalcaemia (HH)	183	3.1
Hypoparathyroidism	0	0
Secondary hyperparathyroidism	262	4.4
Non-PTH hypercalcaemia	5	0.1
Likely normocalcaemic hyperparathyroidism	19	0.3
Normocalcaemic hypoparathyroidism	18	0.3
Normoparathyroid hypercalcemia	88	1.5
Normoparathyroid hypocalcaemia	31	0.5
Unclassified abnormal	37	0.6
Total	**5977**	**100**

Table 2-5: The different patient categories based on their calcium metabolism disorders, after applying the criteria for 25(OH)D<50nmol/L and/or eGFR<60 ml/min/1.73m^2

A further evaluation of their medical notes, excluded fourteen patients due to other exclusion criteria. These were as follows:

Patient number

1. 1236: Excluded – bisphosphonates (zoledronic acid) on index day
2. 1784: Excluded - coeliac disease (positive antibodies, no biopsy performed)
3. 1832: Excluded – PHPT (all other measurements of calcium high)
4. 1849: Included
5. 1977: Excluded – no persistence of high PTH (only one high result)
6. 2136: Excluded - FHH
7. 3584: Excluded – just one measurement of PTH and calcium available
8. 3831: Excluded – just one measurement of PTH available, on bisphosphonates
9. 4103: Included
10. 4213: Excluded – just one measurement of PTH and calcium available
11. 4322: Included
12. 4403: Excluded – bisphosphonates (alendronic acid) on index day
13. 4777: Included
14. 5361: Excluded – loop diuretics (furosemide) on index day
15. 5489: Included
16. 6177: Excluded – treated pseudohypoparathyroidism
17. 6181: Excluded – just one measurement of PTH
18. 6715: Excluded – just one measurement of PTH and calcium available
19. 8483. Excluded – bisphosphonates (aledronic acid) on index day

The number of NPHPT patients identified after applying the exclusion criteria in our referral population was five. All the patients were female and their mean age was 68 years. NPHPT

had a 0.08% prevalence in this population. The consort diagram (Figure 2-7) illustrates the criteria used.

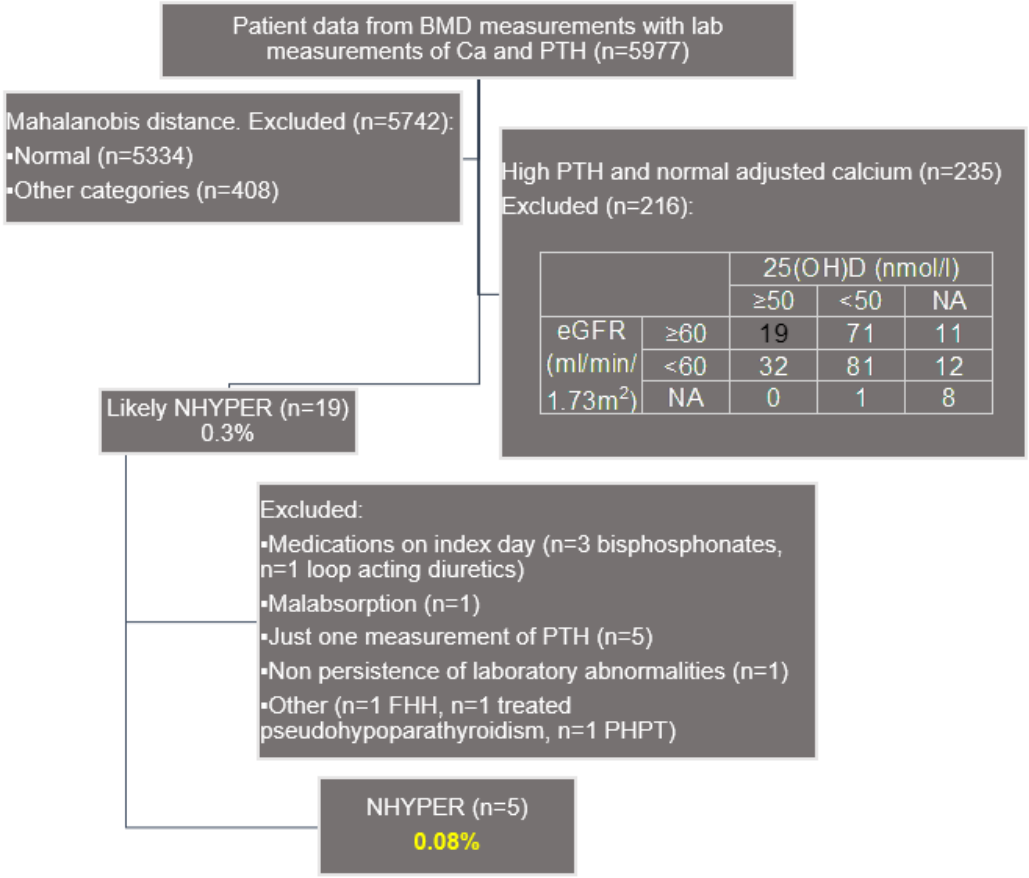


Figure 2-7: Consort diagram illustrating the procedure for identifying the normocalcaemic hyperparathyroidism patients.

Five patients (n=5, prevalence 0.08%) were identified out from a referral population of 5977 patients having both adjusted calcium and PTH

The characteristics of these five patients can be seen in (Table 2-6).

As far as biochemical characteristics of PHPT are concerned, one out of the five NPHPT patients (patient 4322), had a low phosphate level on the index day (0.69 mmol/L) and only

the patient with the highest PTH on the index day (patient 1849, PTH 157 ng/L), had high ALP (148 IU/L).

	Minimum	Maximum	Mean	SD
Age (years)	58	73	68.0	6.00
BMI (kg/m^2)	22.5	44.3	32.22	7.85
PTH (ng/L)	87.0	157.0	109.60	27.75
Adjusted calcium (mmol/L)	2.56	2.58	2.57	0.01
25(OH)D (nmol/L)	56.4	104.6	70.48	19.99
ALP (IU/L)	64.0	148.0	95.40	31.89
Phosphate (mmol/L)	0.69	1.09	0.93	0.17

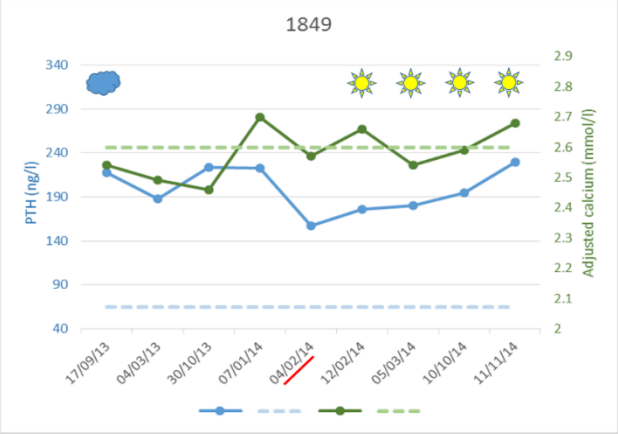
Table 2-6: Characteristics of the normocalcaemic hyperparathyroidism patients (n=5) on the index day

The reasons that the patients were referred and had laboratory evaluations varied. Three out of five of these patients had osteoporosis, the others had osteopenia. Patient 1849 (age 67 years) was referred for a BMD because of “mild primary hyperparathyroidism”; that was also the reason she had the laboratory investigations, as a follow up to her clinic visit. This patient had a fine needle aspiration (FNA) and the findings were compatible with parathyroid gland adenoma. Patient 4103 (age 71 years) was referred because of a previous low trauma fracture and she had laboratory investigations because of a vertebral fracture. She had osteoporosis at the spine. Patient 4322 (age 73 years) had the BMD scan and the laboratory investigations as a follow up for her clinic visit, which she attended because of “mild primary hyperparathyroidism”. She had osteopenia at both the spine and the hip. Patient 4777 (age 58 years) was referred for a BMD because of borderline FRAX score. The laboratory evaluations were performed because BMD was low for age (spine Z score -2.3). This patient

had osteoporosis at both the spine and the hip. Patient 5489 (age 71 years) was referred for a BMD measurement because of “mild primary hyperparathyroidism” and that was the reason she had the laboratory investigations, as a follow up to her clinic visit. She had osteopenia. Due to the limited number of patients identified, there was not sufficient power to perform a statistical analysis in order to evaluate differences in patients’ characteristics amongst the different groups.

The natural history of the five NHPER patients was studied using data from days where both adjusted calcium and PTH were available. The extraction of all the data was done manually, by looking at available results on ICE (the hospital’s laboratory software, having results from 2009). Only the results from 2011 onwards were retrieved, so the follow up period was up to six years (2011-2016). That was because the laboratory changed manufacturer at 2011 and the updated equation could not be applied in measurements performed with a different manufacturer.

Two patterns were identified; intermittent hypercalcaemia and persistent normocalcaemia. Three out of five patients had intermittent hypercalcaemia, two had persistent normocalcaemia; one of them only had two measurements available. The measurement variations were independent of 25(OH)D levels (Figure 2-8).



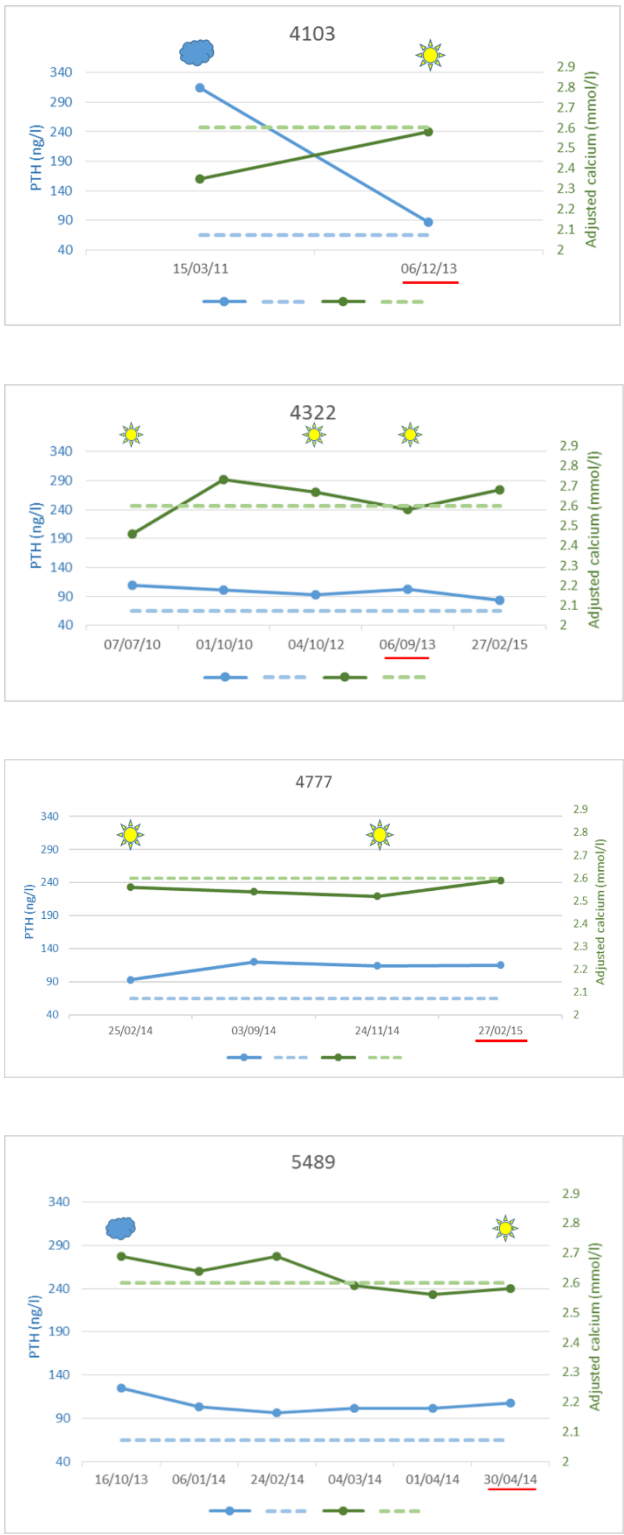


Figure 2-8: Graphs of the adjusted calcium and PTH values for the five normocalcaemic hyperparathyroidism patients throughout time.

Only the calcium measurements that are accompanied by a PTH measurement on the same day are shown. The blue lines indicate PTH levels [dashed= upper limit of normal range (65ng/L), continuous= patient's PTH

variations]. Green lines indicate adjusted calcium levels [dashed= upper limit of normal range (2.60mmol/L), continuous= patient's adjusted calcium variations]. The red line indicates the index day. The sun image indicates 25(OH)D levels≥50nmol/L, whereas the cloud image indicates levels <50nmol/L. Two patients (Subject ID 4103 and subject ID 4777) had persistent normocalcaemia. The calcium variations where independent of the 25(OH)D levels

2.2.1.1.5 Conclusions

The prevalence of NPHPT in the Metabolic Bone Centre referral population, was 0.08% (five patients out of 5977). According to the definition, NPHPT is characterised by persistent elevated PTH measurements and normal calcium. Only two patients out of the whole cohort would really have NPHPT according to the definition; one of them with just two measurements of both calcium and PTH.

2.2.1.2 Problems identified in Analysis 1

After completing analysis 1, several issues were identified. Their description can be found in detail in the Appendix.

2.2.2 Further intermediate analyses

Any other intermediate analyses can be found in the Appendix along with the problems identified and the way they were resolved. Moreover, the final codes used in R studio and their explanations can also be found in the Supplementary material. A summary of the different analyses and their problems can be seen in Table 2-7.

Name	**Number of patients**	**Steps**	**Results for NPHPT**	**Problems**
Analysis 1 Completed 2016	9224 Data 2011-2015 for both prevalence and natural history	1. Mahalanobis distance 2. Categories of patients 3. Exclusion criteria for NPHPT 4. Natural history 2011-2016	19 likely NPHPT Final: 5 patients	1. Assay change in calcium in 2013, could affect results of prevalence 2. Non-reproducible results of MD 3. Problems with database (wrong age, given calculated adjusted calcium)
Analysis 2 Completed 2017	7809 Final 5713 Data 2013-2017	1. Mahalanobis distance 2. Categories of patients 3. Exclusion criteria for NPHPT based on all data	21 likely NPHPT Final: 2 patients	1. Patients identified because of spine T score, underestimation of prevalence
Analysis 3 Completed 2017	8411 Final 6280	1. Mahalanobis distance (final) 2. Categories of patients (final) 3. Exclusion criteria for NPHPT based on all data 4. Natural history 2011-2017	Final: 8 patients	1. Up to now, exclusion due to all available PTH, vitD and eGFR measurements, troublesome. Better to base decision on index date
Analysis 4 Completed 2018	Same as analysis 3 28 likely NPHPT	1. Exclusion criteria on NPHPT based on index date	Final: 15 patients	1. Decided to compare with HH and control 2. ICE not retrieving all the information, to get repeated measurements 3. Wrong to study natural history from 2011, due to change in calcium method
Analysis 5 Completed 2019	Same as analysis 3 28 likely NPHPT	1. Exclusion criteria based on persistence of at least two consecutive measurements 2. Comparison with PHPT and control	Final: 13 patients	1. Decided to adjust the definition of hypercalciuria to 24-hour urine calcium ≥6.25 mmol/24h for women or 7.5 mmol/24h for men and/or ≥ 0.1 mmol/kg/24h
Final analysis Completed 2019	Same as analysis 3	1. Exclusion criteria based on persistence of at least two consecutive measurements	Final: 11 patients	

	28 likely NPHPT	2. Comparison with PHPT and control		

Table 2-7: Summary of the different intermediate analyses on NPHPT and their issues

Chapter 3: Metabolic Bone Centre studies

Section 1: Normocalcaemic hyperparathyroidism, prevalence and natural history

Section 2: Normocalcaemic hypoparathyroidism, prevalence and natural history

Section 3: Further analyses

Section 1: Normocalcaemic hyperparathyroidism

Contribution details

Title	Normocalcemic hyperparathyroidism: study of the prevalence and natural history
Publication status	The material presented in the following section has been written and formatted for submission in The Journal of Clinical Endocrinology and Metabolism (JCEM)
Authors	Marian Schini[1], Richard Jacques[2], Eleanor Oakes[3], Nicola Peel[3], Jennifer Walsh[1], Richard Eastell[1] [1]Department of Oncology and Metabolism, University of Sheffield, UK [2]School of Health and Related Research (ScHARR), University of Sheffield, UK [3]Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT), UK
Authors roles	Study design: MS, RE. Data collection: MS. Data analysis: MS with advice from RJ. Interpretations: all authors. Drafting manuscript: MS. Revising manuscript: all authors. Approval of final manuscript: all authors
Student author's name	Marian Schini Signature

Co-author statement

I hereby declare that I am aware that the work in the manuscript entitled: "The prevalence and natural history of normocalcaemic hypoparathyroidism in a United Kingdom referral population" of which I am a co-author, will for part of the PhD dissertation by the PhD student Marian Schini who made a major contribution to the work stated above.

Co-author name
Richard Jacques
Eleanor Oakes
Nicola Peel
Jennifer Walsh
Richard Eastell

Supervisor confirmation

I have sighted email or other correspondence from all co-authors confirming their certifying authorship.

Name	Signature
Professor Richard Eastell	R Eastell 21/12/19

NORMOCALCEMIC HYPERPARATHYROIDISM: STUDY OF THE PREVALENCE AND NATURAL HISTORY

Marian Schini[1], Richard Jacques[2], Eleanor Oakes[3], Nicola Peel[3], Jennifer Walsh[1], Richard Eastell[1]

[1]Department of Oncology and Metabolism, University of Sheffield, UK

[2]School of Health and Related Research (ScHARR), University of Sheffield, UK

[3]Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT), UK

Short title: NPHPT: Prevalence and Natural History

Keywords: Normocalcemic hyperparathyroidism; prevalence; natural history; epidemiology, primary hyperparathyroidism

3.1.1 Abstract

Context

Normocalcemic hyperparathyroidism (NPHPT) is characterised by persistently normal calcium levels and elevated PTH values, after excluding other causes of secondary hyperparathyroidism. The prevalence of the disease varies a lot and the data on the natural history of this disease are sparse and inconclusive.

Objectives

To describe the prevalence of NPHPT and its natural history in a referral population and to compare the variability of serum calcium with a group of patients with primary hyperparathyroidism (PHPT)

Design

Retrospective study over five years

Setting

Metabolic Bone referral centre

Patients

6280 patients referred for a BMD measurement

Main Outcome Measures

Prevalence and natural history of NPHPT and variability of calcium

Results

We identified NPHPT patients using data from the day of the BMD measurement. We excluded patients with low eGFR or vitamin D, or with no measurements available.

Based on the evaluation of their medical files, we identified eleven patients with NPHPT (prevalence 0.18%). Only four patients had consistent normocalcemia throughout their follow up with only two also having consistently high PTH. None had consistently normal eGFR or vitamin D.

Intermittent hypercalcaemia was present in seven of the eleven NPHPT patients. The mean adjusted calcium was found to be significantly lower in the NPHPT group compared with the PHPT group but higher than the control group. PTH was similar for NPHPT and PHPT. These two groups had similar variability in serum calcium.

Conclusions

NPHPT patients often have episodes of hypercalcemia. We believe that NPHPT is a mild form of primary hyperparathyroidism.

Précis

The prevalence and natural history of patients with normocalcemic hyperparathyroidism were studied. We concluded that the prevalence is low and that these patients probably suffer from PHPT

3.1.2 Introduction

Normocalcemic hyperparathyroidism (NPHPT) is a disorder of calcium metabolism which, despite being mentioned in literature for several years, was only officially defined in 2009, during the Third International Workshop on Asymptomatic Primary Hyperparathyroidism (PHPT) (Eastell et al, 2009). According to the latest guidelines, persistently normal calcium levels (total and ionised) and consistently elevated parathyroid hormone (PTH) values characterise NPHPT. The panel suggested that normal calcium has to be confirmed on several occasions and that an elevated PTH measurement should be confirmed on at least two consecutive measurements. Other causes of secondary hyperparathyroidism have to be excluded; these include medications known to affect PTH levels (diuretics, lithium, denosumab, bisphosphonates, anticonvulsants, phosphorus), low vitamin D, chronic kidney disease (eGFR <60 ml/min/1.73m^2), renal calcium loss (hypercalciuria) and diseases of the gastrointestinal tract known to affect calcium absorption (coeliac disease, inflammatory bowel disease, bariatric surgery) (Eastell et al, 2009; Eastell et al, 2014; Yacobi-Bach et al, 2015).

NPHPT is usually encountered during the laboratory evaluation of osteoporosis and has been associated with consequences known to affect patients with PHPT (accelerated bone loss, kidney stones, higher levels of blood pressure and higher pre-existing glucose intolerance). However, there is no clear evidence for any such associations (Pawlowska & Cusano, 2015; Silverberg et al, 2014).

The prevalence of NPHPT in the literature varies considerably, and it is reported to be between 0.1 and 8.9% (Pawlowska & Cusano, 2015). The reasons for this variation are that different definitions and methodologies were used in the reporting studies; not all causes of secondary hyperparathyroidism were excluded, and some studies did

not test the persistence of high PTH and normal calcium before evaluating the prevalence; thus the results were probably overestimated.

The data on the natural history of this disease are also sparse and inconclusive. Some studies show progression to hypercalcemia, but others fail to show that. The way that many studies identify NPHPT patients is by testing calcium and PTH at baseline, and then they retest the patients after some time to check whether they progressed to hypercalcemia. The variability of calcium in NPHPT during the follow-up period has been poorly described. One study has described the variability as similar to the normal population, whereas patients with PHPT have been described to have more considerable variability in calcium levels (Norman et al, 2011). However, there are not enough data to draw any conclusions.

According to the proceedings of the Fourth International Workshop on Asymptomatic Primary Hyperparathyroidism, NPHPT remains incompletely described, especially regarding its epidemiology, natural history and management (Silverberg et al, 2014). The criteria are not clear concerning how many measurements are needed to establish a diagnosis, which makes the matter complicated for the clinician and confusing for the patient. This study aims to identify the prevalence of NPHPT and study the natural history of this disorder in more detail. It also aims to compare the variability of calcium between PHPT and NPHPT patients.

3.1.3 Materials and methods

3.1.3.1 Study population

The work was performed at the Metabolic Bone Centre (MBC) at Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT) in the United

Kingdom. Data from patients referred for a bone mineral density (BMD) measurement between the 14th January 2013 to the 27th July 2017 were retrospectively evaluated. All the patients included in the database had to have a laboratory evaluation, including calcium and PTH, performed within twenty-eight days of their scan. In general, this department evaluated approximately 650 patients per month at the time of the study and a laboratory workup for secondary osteoporosis was performed in patients having any of the following findings: low BMD for age (Z-score≤ -2.0), vertebral fracture, unexplained bone loss since the previous scan. The day of the laboratory investigations was defined to be the index day. More results of calcium and PTH before and after the index day were retrieved from hospital records and were used to study the natural history of the disease. Only data collected after 14th January 2013 were evaluated because at that point, the laboratory changed the assay for serum calcium, and this would probably affect the accuracy of the results. The end of the follow-up period was the 31st of July 2018.

The analysis was considered a case note review project by Sheffield Teaching Hospitals and did not require a special ethical approval (Project number STH20617).

3.1.3.2 Biochemical measurements

Blood was drawn at any point of the day, so patients were not fasting. All the samples were analysed in the Chemical Pathology Laboratory, STH NHS FT. Intact PTH (second generation) was measured using an immunoassay method by the Roche Cobas 8000 e602 (Roche Diagnostics GmbH, Mannheim, Germany). The interassay coefficient of variation (CV) measured in the laboratory is 2.2 – 3.2% at 34 ng/L, 1.6 –

1.7% at 94 ng/L and 1.4 – 1.8% at 839 ng/L, while the reported reference interval is 15-65 ng/L (1.6 - 6.9 pmol/L).

Serum calcium was measured using a Roche/Hitachi Cobas 8000 e702 automated clinical chemistry analyser (Roche Diagnostics GmbH, Mannheim, Germany). This method uses 5-nitro-5'-methyl-BAPTA (NM-BAPTA) reagent. The interassay coefficient of variation as measured in the laboratory is 1.1 – 1.5% at 1.52 mmol/L and 0.6 – 1.1% at 3.07 mmol/L. Albumin measurement was performed using a Roche/Hitachi Cobas 8000 e702 analyser (Roche Diagnostics GmbH, Mannheim, Germany). The interassay coefficient of variation as measured by the laboratory is 1.5 – 2.4% at 33.9 g/L and 1.0 – 1.7% at 59.7 g/L. The albumin-adjusted calcium was used for this study and was calculated using the following equation based on data from the local laboratory.

$$Adjusted\ Ca = Total\ Ca + [0.0172(43 - Albumin)]$$

The reference interval for adjusted calcium was 2.20-2.60 nmol/L (8.8-10.4 mg/dL).

25(OH)D was measured using the IDS-iSYS automated immunoassays (Immunodiagnostic Systems, Boldon, UK) until May 2013. The interassay coefficient of variation reported by the manufacturer was 10.4% at 64.5 nmol/L. The assay then changed to a competitive binding protein one and was performed by Roche modular analytics Cobas E170 (Roche Diagnostics GmbH, Mannheim, Germany). The laboratory interassay coefficient of variation for this assay was 6.5 – 9.9% at 48.2 nmol/L and 4.5 – 6.3% at 92.3 nmol/L. The Sheffield Teaching Hospitals laboratory was participating in the UK Vitamin D External Quality Assessment Scheme (DEQAS) at the time of this study.

Creatinine was measured using a Roche/Hitachi Cobas c8000 e702 analyser (Roche Diagnostics GmbH, Mannheim, Germany). The interassay coefficient of variation for this assay was 2.7 – 6.1% at 55.7 μmol/L and 2.3 – 4.0% at 458 μmol/L. The equation used to calculate eGFR was the Modification of Diet in Renal Disease (MDRD) Study equation:

$$eGFR\left(\frac{mL}{min \times 1.73\ m^2}\right) = 175 \times (\text{Serum creatine})^{-1154} \times age^{-0.203} \times [0.742\ if\ female] \times [1.212\ if\ black]$$

This equation changed in August 2015; the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation has been in use since then.

$$eGFR\left(\frac{mL}{min \times 1.73\ m^2}\right) = 141 \times \min\left(\frac{Scr}{k}, 1\right)^{\alpha} \times \max\left(\frac{Scr}{k}, 1\right)^{-1.209} \times 0.993^{age} \times 1.018\ [if\ female] \times 1.159\ [if\ black]$$

Scr is serum creatinine in mg/dL, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, max indicates the maximum of Scr/κ or 1 (KDIGO, 2012).

Alkaline phosphatase was measured using a Roche/Hitachi Cobas c8000 e702 analyser. The interassay coefficient of variation for this assay was 2.4% at 92.8 IU/L and 1.7% at 224 IU/L. The same analyser was used for the measurement of serum phosphate and the interassay coefficient was 1.4% at 1.23 mmol/L and 1.2 at 2.04 mmol/L.

Bone mineral density was performed using dual-energy X-ray absorptiometry (DXA) of the lumbar spine and the proximal femur in posteroanterior projection. At the time of the study, there were three Hologic (Waltham, MA) DXA scanners within the centre. The least significant change (LSC) used in the MBC was 4.5% for both the spine and the hip. There were three scanning rooms in the department and each room has a different scanner. Room A: Hologic Discovery A in use since 2010. Room B: Hologic

QDR4500A was in use from 2011 until the 11th September 2013. The scanner was then decommissioned and replaced by a Hologic Discovery SL. Room C: Hologic Delphi C was in use until the 3rd November 2014. The scanner was then decommissioned and replaced by a Hologic Horizon A. The different DXA scanners were cross-calibrated upon installation.

3.1.3.3 Statistical analysis

As calcium and PTH are not independent variables, a bivariate statistical approach was used to define the different categories of patients. Mahalanobis distance is a multi-dimensional generalization of the concept of measuring the number of standard deviations (SD) away an observation is from the mean of a distribution. It is a classical method of defining outliers in a multivariate point cloud and is defined for each point x_i, by the following equation, where MD_i: Mahalanobis distance, μ: arithmetic mean of the dataset and S: sample covariance matrix.

$$MD_i^2 = (x_i - \mu)^T S^{-1} (x_i - \mu)$$

The distance MD_i gives the distance of point x_i from the centre of the cluster of points, taking into account the shape of the cluster. Observations are considered outliers if $MD^2 > X^2_{2;0.975} = 7.378$ (97,5th percentile of chi-squared distribution with two degrees of freedom) (Rousseeuw & van Zomeren, 1990; Van Aelst & Rousseeuw, 2009). The correlation analysis of adjusted calcium and PTH was performed based on a log10 transformation of the two variables. A similar approach has been used in the past to evaluate the prevalence of NPHPT in a different group of patients. Using this method, subjects were identified as "normal" if they were inside the ellipse and "abnormal" if they were outside. In order to get the different categories described below, they used

the reference interval for adjusted calcium. However, they used the geometric mean of the reference interval for PTH (Palermo et al, 2015). We have adjusted this method and brought it closer to the everyday clinical practice, by using the clinical reference intervals for both adjusted calcium and PTH. Doing so, patients were divided into ten categories as described below. "High" or "low" refer to values of either adjusted calcium or PTH being above or below the reference interval, respectively.

To compare the variability of different analytes in the three groups, the overall mean and the pooled within-subject standard deviation of each analyte was calculated. The analysis of variance method was used to calculate the within subject variance, as this method deals with the case of subjects having different numbers of observations. To follow this method, we first checked the assumption that the standard deviation was unrelated to the magnitude of the measurement. This was done graphically, by plotting the individual subjects' standard deviations against their means and analytically by calculating a rank correlation coefficient (Bland & Altman, 1996a).

To compare characteristics between the groups at baseline, one-way ANOVA and Kruskal-Wallis were used to compare the quantitative data. The results are presented as mean and standard deviation when ANOVA was used for the analysis, as geometric mean and 95% confidence intervals when log transformation was performed before analysis with ANOVA or as median and interquartile range (IQR) in the case where non parametric tests were used for the analysis. The Mann-Whitney test was used to compare the 24-hour urine calcium between the NPHPT and PHPT groups. For the comparison of the overall measurements of PTH and adjusted calcium resulting from follow up, we used a mixed linear model. Individual variability was modelled using a random effect and the difference between groups was estimated using a fixed effect.

The statistical analyses have been performed using the R studio statistical software version 1.1.442 (RStudio, Inc. Boston).

3.1.3.4 Definitions of the different groups

As mentioned in the statistics section, patients were divided into ten categories based on their laboratory intervals for adjusted calcium and PTH. The cut-off for vitamin D deficiency was 50 nmol/L according to the Royal Osteoporosis Society guidelines (Aspray et al, 2014). The categories were defined as follows. Normal: anyone inside the ellipse; the rest of the groups described were outside the ellipse. Hyperparathyroid hypercalcemia (HH) included patients with PHPT and familial hypocalciuric hypercalcemia (FHH) and was defined as anyone outside the ellipse having high adjusted calcium and high PTH. Hypoparathyroidism: low adjusted calcium and low PTH. Secondary hyperparathyroidism: a) low adjusted calcium and high PTH or, b) normal adjusted calcium and high PTH with 25(OH)D<50nmol/L or eGFR<60 ml/min/1.73m^2. Non-PTH hypercalcemia: high adjusted calcium and low PTH. Normocalcemic hyperparathyroidism (NPHPT): normal adjusted calcium and high PTH, given that 25(OH)D≥50 nmol/L and eGFR≥60 ml/min/1.73m^2. Normocalcemic hypoparathyroidism (NHYPO): normal adjusted calcium and low PTH. Normoparathyroid hypercalcemia: high adjusted calcium and normal PTH. Normoparathyroid hypocalcemia: low adjusted calcium and normal PTH. Unidentified: normal adjusted calcium and normal PTH, but being outside the ellipse. The groups of interest for this study were the normal population and the patients with HH and NPHPT. In order to characterise these groups further, the following procedures were followed.

For the NPHPT patients identified from the index date, a careful evaluation of their medical records and other laboratory investigations were performed in order to check for exclusion criteria according to the guidelines (see introduction). Renal calcium loss was defined as having 24-hour urine calcium ≥6.25 mmol/24h for women or 7.5 mmol/24h for men and/or ≥ 0.1 mmol/kg/24h (Worcester & Coe, 2008). Moreover, and in order to be in line with the international guidelines on persistence, only patients with persistently normal adjusted calcium and elevated PTH on two consecutive occasions were included.

For the hyperparathyroid hypercalcemia patients (HH), only the patients with vitamin D ≥50 nmol/L and eGFR≥60 ml/min/1.73m^2 on the index date were chosen to be used for the variability analysis. An evaluation of their medical files excluded patients with other diseases like renal transplant. Results after parathyroid surgery were excluded. Once again, only patients with consistently high calcium and PTH on at least two consecutive occasions were included. In order to only include patients with PHPT (and to exclude FHH) in this group, the patients had to have one or more of the following criteria: 24-hour urine calcium> 2.5 mmol/24h or fractional excretion >0.02; surgically proven PHPT (correction of high calcium after parathyroid surgery). At this point, all remaining patients in this group were defined as primary hyperparathyroidism (PHPT).

For the control population, a random sample of 300 subjects from inside the ellipse having normal eGFR and being vitamin D replete (as defined above) on the index date was chosen.

3.1.4 Results

In total, 6293 patients attended the Metabolic Bone Centre and were assessed for secondary osteoporosis from January 2013 to July 2017. All these patients had a BMD measurement and a laboratory evaluation. Thirteen patients did not have PTH available on the index day and were excluded. The total number of patients analysed was 6280; their mean age was 66 years (range 16-100), and 4527 (72%) were female. After applying the Mahalanobis distance, the ellipse seen in Figure 3-1 was drawn. In total, 5574 patients were inside the ellipse and were considered as “normal” while the rest were outliers and were divided into disease categories using the laboratory reference interval for calcium and PTH (Table 3-1).

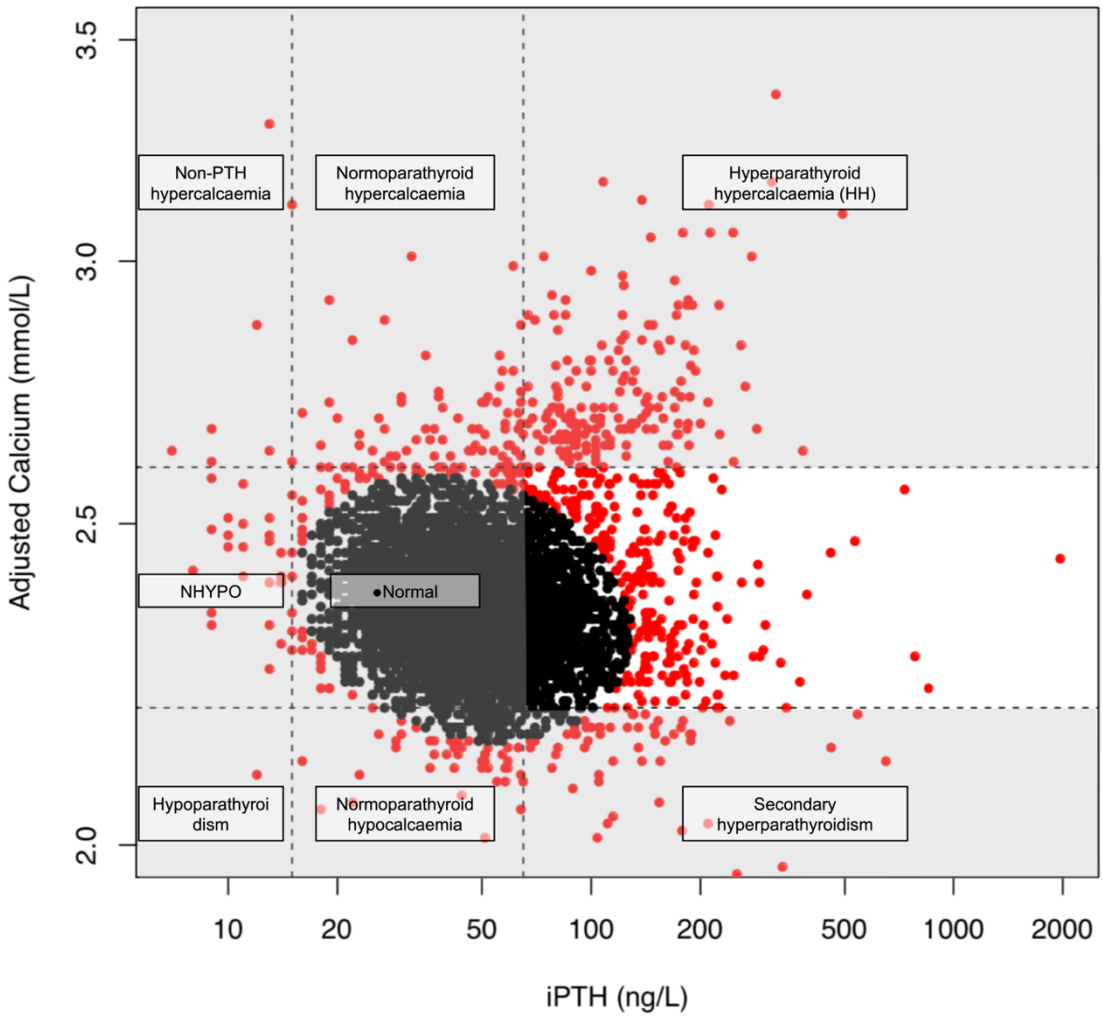


Figure 3-1: Data results from adjusted calcium and PTH.

The ellipse was formed using a statistical method (Mahalanobis distance) to identify "normal" subjects (black dots) and "abnormal" ones (red dots). The reference interval of both adjusted calcium and PTH (horizontal and vertical dashed lines, respectively), were used to identify patient categories. The definitions used are described in the methods section. The white area includes patients with normal adjusted calcium and high PTH (n=265); these were either potentially NPHPT patients [given that 25(OH)D≥50nmol/L and eGFR≥60 ml/min/1.73m^2] or secondary hyperparathyroidism patients [given that 25(OH)D<50nmol/L and/or eGFR<60 ml/min/1.73m2]. NPHPT: Normocalcemic hyperparathyroidism. NHYPO: Normocalcemic hypoparathyroidism

Categories	Number (%)
Normal	5574 (88.76)
Hyperparathyroid hypercalcemia (HH)	172 (2.74)
Hypoparathyroidism	1 (0.02)
Secondary hyperparathyroidism	291 (4.63)
Non-PTH hypercalcemia	6 (0.10)
Normocalcemic hyperparathyroidism (NPHPT)	28 (0.45)
Normocalcemic hypoparathyroidism (NHYPO)	22 (0.35)
Normoparathyroid hypercalcemia	67 (1.07)
Normoparathyroid hypocalcemia	43 (0.68)
Unclassified abnormal	76 (1.21)

Table 3-1: The different patient categories based on their calcium metabolism disorders.

The following definitions were used. Normal: anyone inside the ellipse; the rest of the groups described were outside the ellipse. Hyperparathyroid hypercalcemia (HH): high adjusted calcium and high PTH. Hypoparathyroidism: low adjusted calcium and low PTH. Secondary hyperparathyroidism: low adjusted calcium and high PTH or, b) normal adjusted calcium and high PTH with 25(OH)D<50nmol/L or eGFR<60 ml/min/1.73m^2. Non-PTH hypercalcemia: high adjusted calcium and low PTH. Normocalcemic hyperparathyroidism (NPHPT): normal adjusted calcium and high PTH, given that 25(OH)D≥50nmol/L and eGFR≥60 ml/min/1.73m^2. Normocalcemic hypoparathyroidism (NHYPO): normal adjusted calcium and low PTH. Normoparathyroid hypercalcemia: high adjusted calcium and normal PTH. Normoparathyroid hypocalcemia: low adjusted calcium and normal PTH. Unclassified abnormal: normal adjusted calcium and normal PTH, but being outside the ellipse.

In total, 28 patients initially fulfilled the inclusion criteria for NPHPT (normal calcium, high PTH, both on at least two occasions, normal eGFR and vitamin D replete on index day). A careful evaluation of their medical records and their medical history excluded 17 patients (Table 3-2). The natural history was then studied, using calcium measurements available from January 2013 to the end of July 2018. Two patients

were excluded because of PTH inconsistency (S2406 and S3820). In total, 11 patients were identified as having NPHPT with confirmed persistent results of normal calcium and elevated PTH on at least two occasions. The prevalence was 0.18% (95% CI 0.10 - 0.31). The mean age of these patients was 69 years, and 91% were female. None had parathyroid surgery.

Study number	Age (years)	Gender	Medical files check	Persistence of calcium and PTH	Natural history of adjusted calcium
S0189	65	F	Excluded - anticonvulsants		
S0227	59	F	Included	Yes	Persistent normocalcemia
S0449	65	F	Excluded - treated pseudohypoparathyroidism		
S0567	72	F	Excluded - tertiary hyperparathyroidism-renal transplant		
S0696	75	F	Excluded - bisphosphonates		
S0757	68	F	Included	Yes	Intermittent hypercalcemia
S0871	83	M	Excluded - bisphosphonates		
S0911	70	F	Included	Yes	Persistent normocalcemia
S1194	74	F	Excluded - bisphosphonates		
S1620	75	F	Included	Yes	Persistent normocalcemia
S1692	79	F	Excluded - bisphosphonates		

S1753	86	M	Included	Yes	Intermittent hypercalcemia
S2406	67	F	Included	No	
S2453	57	F	Included	Yes	Intermittent hypercalcemia
S2654	88	F	Excluded - Crohn's-bisphosphonates-furosemide		
S2720	83	F	Included	Yes	Intermittent hypercalcemia
S3021	64	F	Included	Yes	Persistent normocalcemia
S3703	72	F	Excluded- anticonvulsants		
S3812	70	F	Included		Intermittent hypercalcemia
S3820	49	F	Included	No	
S3841	84	F	Excluded - bisphosphonates		
S3882	69	F	Excluded - hypercalciuria		
S4392	66	F	Included	Yes	Intermittent hypercalcemia
S4618	59	F	Included	Yes	Intermittent hypercalcemia
S4903	59	M	Excluded - renal transplant		
S5321	78	F	Excluded - bisphosphonates		
S5369	64	F	Excluded - hypercalciuria		
S5408	82	F	Excluded - furosemide		

Table 3-2: Patients checked for inclusion in the normocalcemic hyperparathyroidism group (NPHPT).

F: female; M: male

Two patterns were identified while studying the natural history; persistent normocalcemia and intermittent hypercalcemia (Figure 3-2), (Figure 3-3). Intermittent hypercalcemia occurred in seven patients. Persistent normocalcemia was rare and only occurred in four patients (0.06% of the whole population). However, only two of these patients (S0227 and S1620) had consistently high PTH, but they were not consistently vitamin D replete and/or did not have consistently normal eGFR. If the international guidelines were strictly applied, the prevalence of NPHPT in this population would be zero.

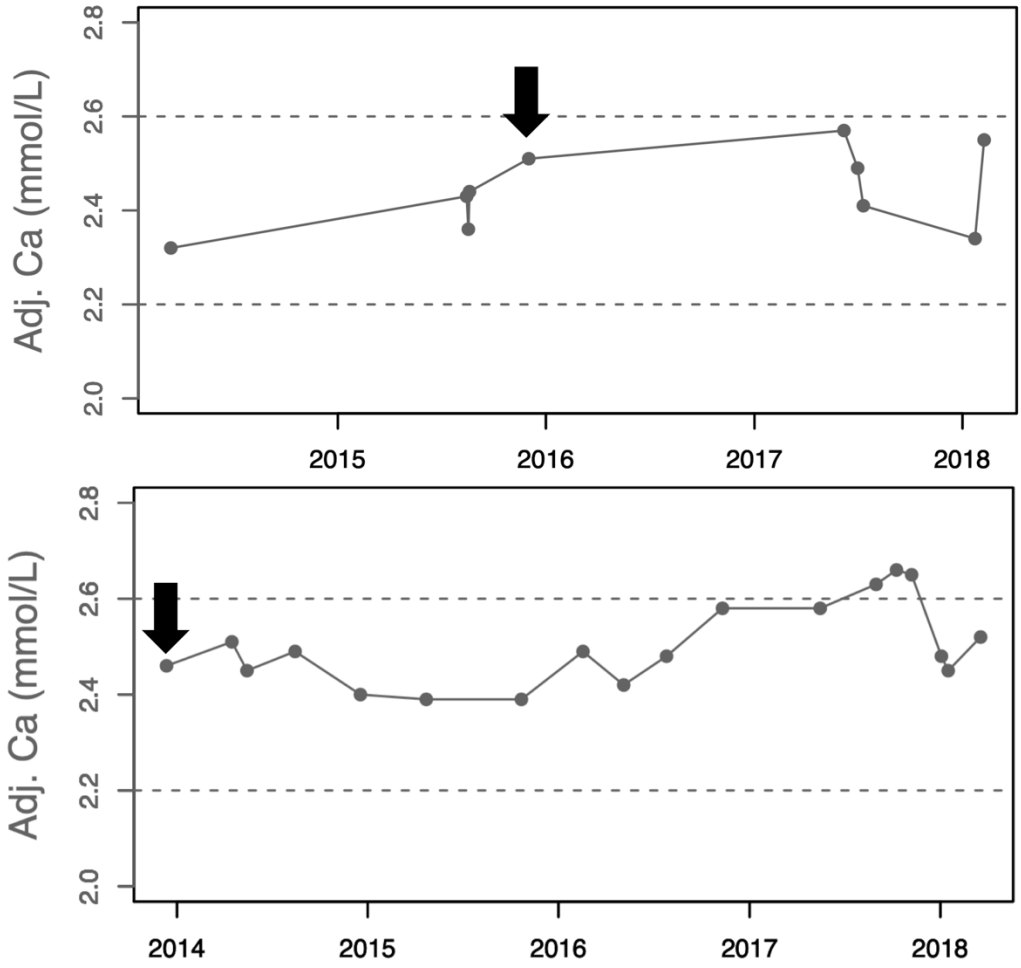


Figure 3-2: The two patterns identified in patients with normocalcemic hyperparathyroidism (NPHPT) when studying the natural history of adjusted calcium.

Persistent normocalcemia (top figure, patient S1620) and, intermittent hypercalcemia (bottom figure, patient S2720). The arrows represent the index day (day of bone mineral density scan). The x-axes represent the year of follow up. Adj.Ca: adjusted calcium.

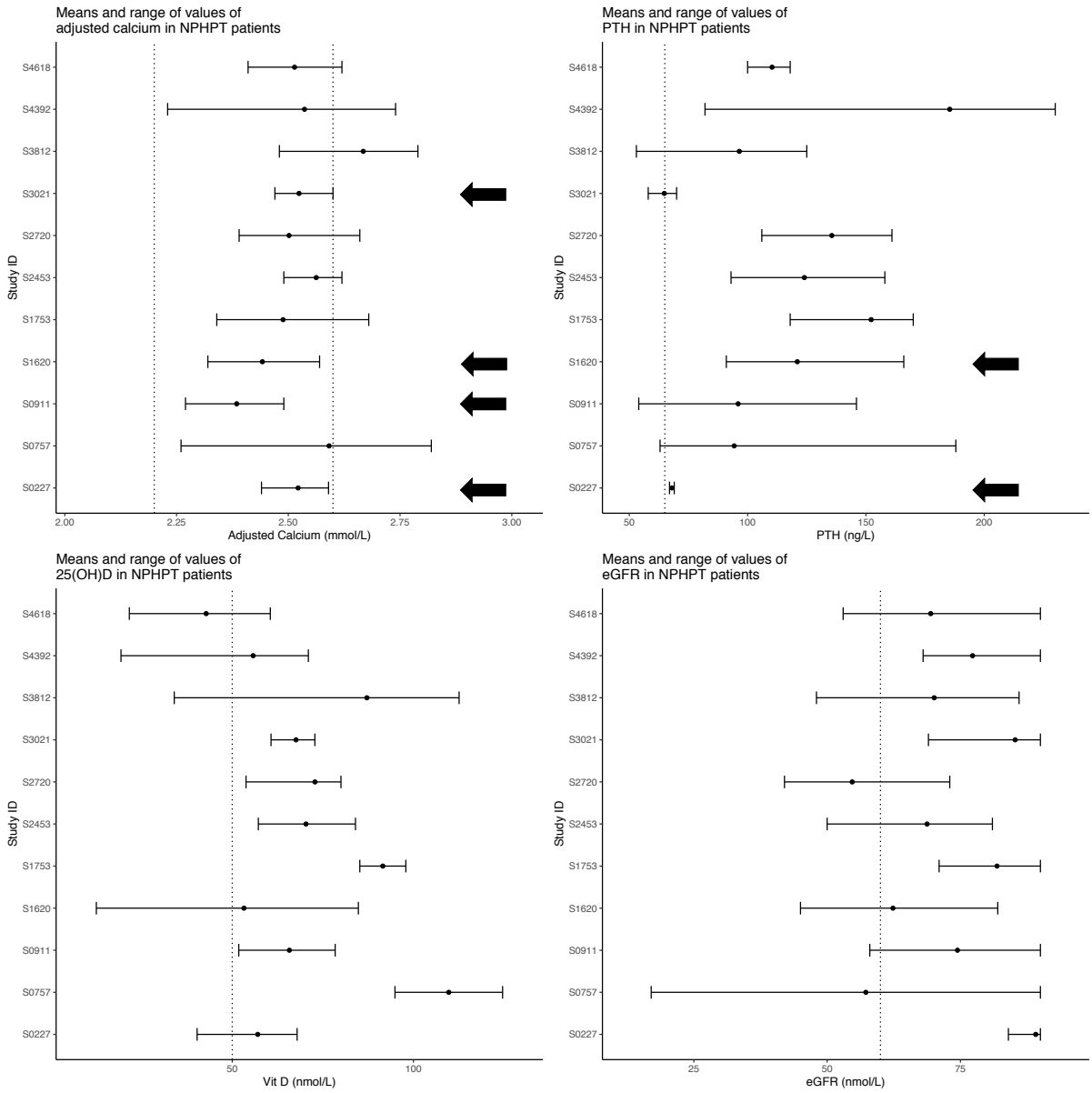


Figure 3-3: Means and range of values of different analytes in the 11 NPHPT patients.

The dashed lines represent the reference interval for adjusted calcium, the upper reference interval for PTH and the desired level for 25(OH)D and eGFR. Persistent normocalcemia was rare, only occurred in 4 patients (shown in the graph with arrows). However, only two patients (S0227 and S1620) had consistently high PTH. These patients were not consistently vitamin D replete and/or did not have consistently normal eGFR. PTH: parathyroid hormone, NPHPT: Normocalcemic hyperparathyroidism. PHPT: Primary hyperparathyroidism

Out of the 172 patients identified with HH, 29 were vitamin D replete and had normal eGFR on the index date. The evaluation of their medical records excluded one patient due to previous renal transplant (S2706). One patient had their index date after their parathyroid surgery and was also excluded (S5826). Eight patients were excluded because they only had one result for PTH, and the persistence could not be confirmed, while one patient was excluded because of not having at least two measurements of calcium consistently high. One patient was excluded because FHH could not be ruled out due to lack of urine calcium (S2031). The final PHPT group had 17 patients (mean age 69, 89% female). Twelve patients had persistent hypercalcemia (70%), while the remaining six had intermittent hypercalcemia, a pattern also seen in patients with NPHPT.

The control population group consisted of 300 subjects (mean age 67 years, 71% female). There was no statistically significant difference in age or gender distribution between the groups (all p values>0.05).

The baseline characteristics on the index date are summarised in Table 3-3. The three groups had significant differences in adjusted calcium on the index date. PTH did not differ between the NPHPT and PHPT groups. The groups had similar age and gender-adjusted BMD results. Phosphate was significantly lower in the PHPT group compared to the control group. All the other variables studied did not have any differences between the three groups at baseline.

	Control (n=300)	**NPHPT (n=11)**	**PHPT**	**p value**

			(n=17)	
Female (%)	214 (71)	10 (92)	15 (88)	0.122
Age (years)[a]	70 (20)	68 (11)	67 (6)	0.975
BMI (g/cm^2)[c]	25.6 (25.0, 26.3)	30.1 (24.4, 34.0)	26.2 (23.4, 28.9)	0.303
PTH (ng/L)[c]	42.5 (40.8, 44.2)	106.8 (86.9, 123.9)	102.4 (89.0, 112.4)	**<0.001**
Adjusted calcium (mmol/L)[b]	2.37 (0.08)	2.55 (0.05)	2.75 (0.11)	**<0.001**
Phosphate (mmol/L)[b]	1.12 (0.18)	1.04 (0.14)	0.89 (0.16)	**<0.001**
Alkaline phosphatase (IU/L)[a]	78 (37)	98 (33)	88 (27)	o.070
25(OH)D (nmol/L)[a]	78.9 (32.9)	62.8 (23.5)	71.4 (30.5)	0.083
Z score spine [b]	-0.1 (1.7)	0.2 (2.2)	-0.2 (1.3)	0.932
Z score neck [b]	-0.4 (1.0)	-0.1 (1.3)	-0.4 (0.8)	0.770

Table 3-3: Characteristics of the three groups on the index date.

[a] shown as median (interquartile range); [b] shown as mean (standard deviation); [c] shown as geometric mean (95% confidence interval). The results of the pairwise comparisons were as follows: PTH in the control group differed significantly from both the NPHPT and PHPT group, but there was no statistically significant difference between the NPHPT and PHPT groups. Adjusted calcium differed significantly between all the groups. Phosphate differed significantly only between the PHPT and control group. BMI: body mass index; PTH: parathyroid hormone; NPHPT: normocalcemic hyperparathyroidism; PHPT: primary hyperparathyroidism; n= number of patients

After taking all the follow-up measurements into account, the mean adjusted calcium was still found to be significantly lower in the NPHPT group compared with the PHPT group (2.52 and 2.74 mmol/L respectively). The control group had significantly lower adjusted calcium levels from all the other groups (2.35 mmol/L, all pairwise

comparisons p<0.001). PTH did not differ significantly between NPHPT and PHPT (115.4 and 116.0 ng/L respectively) but was significantly higher than in the control group (48.1 ng/L, p<0.001) (Figure 3-4). The PHPT and NPHPT groups had similar calcium variability [within-subject SD (95% CI): 0.088 (0.079, 0.097) and 0.089 (0.080, 1.000) mmol/L respectively]. The variability of calcium in the control group was slightly smaller than PHPT and NPHPT [within-subject SD 0.083 (0.080, 0.085) mmol/L]. The 24-hour urine calcium was higher in the PHPT group compared to the NPHPT groups (mean 7.51 and 4.25 mmol/24hour respectively) but the difference was not significant (p 0.063).

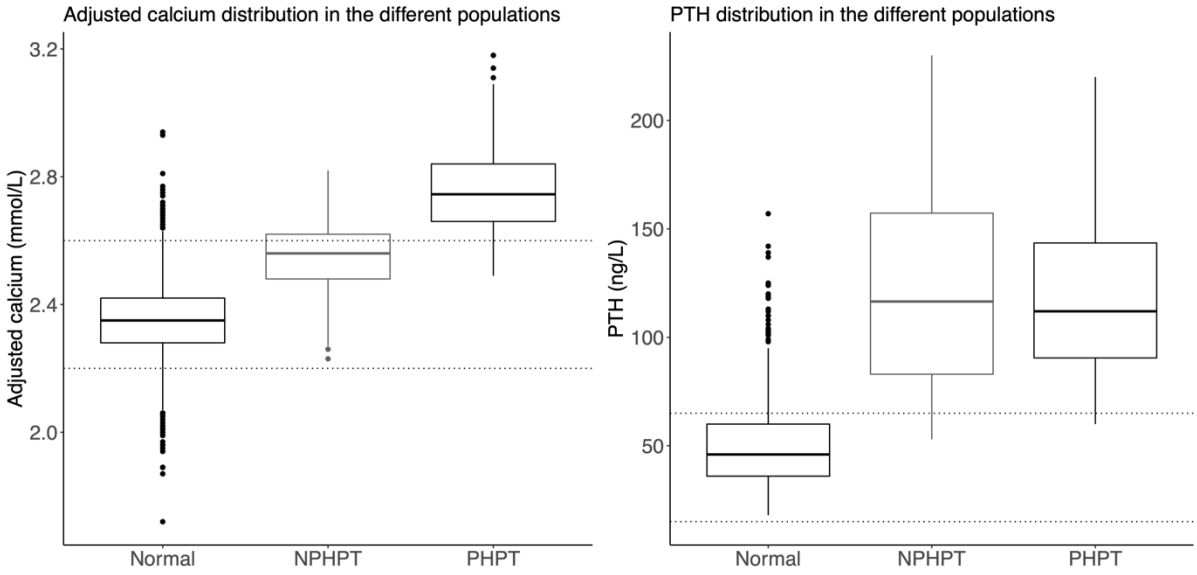


Figure 3-4. Boxplots showing the variability of calcium and PTH in the normal population and patients with PHPT and NPHPT.

The mean adjusted calcium was found to be significantly lower in the NPHPT group compared with the PHPT group (p<0.001). The normal group had significantly lower adjusted calcium levels from all the other groups (p<0.001). PTH did not differ significantly between these groups but was significantly

higher from the normal population (p<0.001). PTH: parathyroid hormone, NPHPT: Normocalcemic hyperparathyroidism. PHPT: Primary hyperparathyroidism

3.1.5 Discussion

The international guidelines suggest that patients with NPHPT should have consistently normal values of calcium. Moreover, PTH should be high on at least two to three consecutive measurements. However, we observed in this population that persistent normocalcemia is rarely the case and using an index day to identify NPHPT patients can lead to overestimation of the prevalence. Patients with NPHPT usually have intermittent hypercalcemia as do PHPT patients, and we believe that NPHPT is a variant of PHPT. The intermittent nature of serum calcium in both NPHPT and PHPT results from the relatively high variability of serum calcium in all patient groups.

The studies available for the prevalence of NPHPT use different definitions and patient populations. Often the baseline data are the only ones analysed, without evaluation of persistence. In order to study the natural history, only one further measurement during follow up is used and the variability of calcium in the follow-up period is not described.

To our knowledge, two studies have reported the prevalence of NPHPT from referral centres (Table 3-4), one of them having the highest prevalence in the literature (8.9%) (Marques et al, 2011). The mean adjusted calcium was similar between the NPHPT group and the rest of the patients, a pattern not seen in our study. They also reported higher levels of PTH in the NPHPT group [109.5 (45.2) vs 39.1 (14.3) pg/mL, p <0.001] (Marques et al, 2011). Another estimate of the prevalence from a referral centre is from a group in the Czech Republic. They report 187 patients (1.2%) with NPHPT

(Siprova et al, 2016). The prevalence of NPHPT was also studied in the general population and was reported to be between 0.1 and 6% (Table 3-4).

The results for the natural history remain controversial with some studies reporting no conversion to hypercalcemia (Ayturk et al, 2006; Garcia-Martin et al, 2012; Tordjman et al, 2004), while others report a small percentage of patients becoming hypercalcemic (Cusano et al, 2013a; Diri et al, 2014; Kontogeorgos et al, 2015; Silverberg & Bilezikian, 2003; Siprova et al, 2016). The problem with the studies on natural history is that, although they might check persistence at baseline on two to three occasions, they then check the laboratory measurements on only one occasion after a certain period; if at that point the patient has high calcium, they conclude that the patient has progressed to hypercalcemia. All the studies are summarised in Table 3-5. Silverberg et al. found hypercalcemia in three patients after one year of follow up. The researchers from this paper first proposed that NPHPT is the first phase of a biphasic disease course, which can be followed by hypercalcemic hyperparathyroidism (Silverberg & Bilezikian, 2003). The group from the Czech Republic followed the 187 patients with NPHPT for one to seven years. Out of them, 151 patients (81%) remained normocalcemic, while 36 (19%) became hypercalcemic; 24 (67%) increased their calcium to high levels within two years, 10 (28%) within 2 to 4 years and 2 (5%) after more than four years. They also reported that 23 patients out of the 36 patients (64%) had persistent hypercalcemia, while 13 (36%) had intermittent hypercalcemia, a pattern also observed in our study. The baseline calcium of patients that remained normocalcemic was significantly lower than the ones that became hypercalcemic (Siprova et al, 2016). A more recent study evaluating NPHPT patients going through parathyroidectomy, reported that 40.8% of these patients had episodes of hypercalcaemia >1 year prior to surgery (Sho et al, 2019).

We believe that NPHPT is a variant of PHPT. This issue has been recently addressed by the first European Society of Endocrinology Workshop (PARAT). In their consensus statement, they mentioned that the individual variation in total calcium is four times narrower than the population reference interval and the inter individual variability. In order to make sure that a person has normal calcium or not, we need to know their individual range of variability. There is a chance that an individual might have an elevated calcium in comparison with their individual range, but still within the normal population reference interval for calcium. These patients should probably be characterised as having primary hyperparathyroidism and not normocalcaemic hyperparathyroidism and be followed up and treated accordingly. This is of course after excluding causes of secondary hyperparathyroidism (Bollerslev et al, 2019).

None of our patients progressed to persistent hypercalcemia; the patterns observed were either persistent normocalcemia or intermittent hypercalcemia. The four patients with persistent normocalcemia did not fulfil all the other criteria according to the international guidelines. Our hypothesis is supported by the fact that the underlying pathology of NPHPT seems to be similar to PHPT. A few studies report that multiglandular disease is more common in NPHPT (Koumakis et al, 2013; Lim et al, 2017; Pandian et al, 2019; Traini et al, 2018) but not all reach statistical significance (Gómez-Ramírez et al, 2019; Kiriakopoulos et al, 2018; Yu et al, 2019). However, patients included in these studies probably had secondary hyperparathyroidism resulting from vitamin D deficiency and chronic kidney disease (Lim et al, 2017; Pandian et al, 2019). Most studies report that the average adenoma weight was lower in the NPHPT group compared to the PHPT group of patients (Kiriakopoulos et al, 2018; Koumakis et al, 2013; Maruani et al, 2003).

This hypothesis, however, is not fully supported by recently published genetic data on NPHPT. Based on the hypothesis that NPHPT can be caused by PTH resistance (Maruani et al, 2003), and the fact that the single nucleotide polymorphism (SNP) rs17251221 (A986S) in the CaSR has been linked to PTH resistance, researchers in Spain recently investigated the effect of this SNP to NPHPT patients. They recruited and prospectively studied 61 consecutive patients with NPHPT and asymptomatic PHPT. These patients had a follow up of one year to check for the persistence of their laboratory investigations on at least two occasions. There were 38 patients (n=24 NPHPT and n=14 PHPT) with the wild type genotype A986A and 23 S allele carriers (n=20 A986S or n=3 S986S). Seventeen NPHPT patients were S allele carriers. In these patients, the S allele was associated with significantly higher levels of serum intact PTH (P=0.024) after adjusting for factors like vitamin D and calcium concentrations. There was no association with the S allele in PHPT patients (Diaz-Soto et al, 2015).

In our series, we found similar variability of adjusted calcium in patients with NPHPT and PHPT. The variability of calcium and PTH has been studied previously in a prospective series of surgically proven PHPT. In this study (Norman et al, 2011), the definition of NPHPT was every measurement of calcium being below 10.2 mg/dL (2.55 mmol/L), and only 1.1% of the patients fulfilled this criterion. Almost all the patients with PHPT had variable calcium levels, with 74% having intermittent hypercalcemia within the previous year. This study did not include a control group but mentioned that serum calcium was more variable in PHPT patients compared to the same patients before developing this disorder. The variability before was described to be 0.19 (0.09) mg/dL [0.0475 (0.0225) mmol/L]. After conversion to PHPT, the variability more than doubled [0.1 (0.0825) mmol/L] with calcium varying by more than 1mg/dL (0.25

mmol/L) from month to month in some cases. The authors suggested that this could be a loss of calcium homeostasis by a parathyroid tumour. Patients with normocalcemic hyperparathyroidism were reported as an exception, presenting with minimal variability, like the one in the patients before PHPT (Norman et al, 2011). Minimal variability is not the finding from our study, as both NPHPT and PHPT patients had similar calcium variability.

The variability described in calcium in these patients complicates their monitoring. According to the international guidelines, calcium and PTH should be tested every year, while a BMD check should be performed every one to two years. If there is a progression to hypercalcemia, patients should be treated according to the guidelines on asymptomatic primary hyperparathyroidism (Bilezikian et al, 2014). However, a majority of these patients have intermittent hypercalcemia; the subsequent results might be confusing for both the care provider and the patient on whether surgery would be recommended.

We used a statistical approach to define the different categories in this population. Our results showed that a large percentage of subjects were considered “normal” (inside the ellipse) using this approach, even though they had elevated PTH according to the reference interval. The subjects that were normal but had higher PTH than the reference interval, were found to be significantly older than the ones that were normal and also had normal PTH according to the current reference interval (data not shown). These results align well with the fact that PTH is known to increase with age. This increase was recently found to be independent of 25(OH)D, ionised calcium, phosphate and renal function (Carrivick et al, 2015). It has been previously suggested that age-related PTH reference intervals should be established (Eastell et al, 2014). Our study supports this statement. This adjustment for the range becomes more

relevant now that normocalcaemic hyperparathyroidism is further described in literature. By using the current reference interval in everyday practice we are probably over-diagnosing NPHPT and may be subjecting older individuals to unnecessary testing. An increase of the upper limit of the reference interval in PTH could alter the number of patients diagnosed with this disorder.

This study describes the largest population ever studied to identify NPHPT and provides data during a long follow up period (five years). There are several limitations to this study. The samples were not fasting and ionised calcium was not available. The methods for vitamin D and eGFR changed during the follow up period and that might have affected some results. We have used a 25(OH)D threshold of 50 nmol/L for identifying high PTH due to D deficiency, but the cross-sectional data indicate that values between 50 and 75 nmol/L might be associated with slight elevations of PTH thus result in overdiagnosis of NPHPT. Many researchers recommend using a higher cut-off of 75 nmol/L. We have used the cut-off advised by the Royal Osteoporosis Society guidelines and the Fourth International Workshop on Asymptomatic Primary Hyperparathyroidism (Aspray et al, 2014; Eastell et al, 2014). The impact of implementing the CKD-EPI equation is that it may have improved the accuracy in patients with an eGFR of 50-60 ml/min/1.73 m^2 and might have resulted in fewer people being clustered as CKD. However, it might have increased the number of older people having CKD. The equation used to calculate the pooled SD cannot really distinguish between variations due to the progression of the disease or those due to natural variation. We assume that when taking measurements close to each other, then the pooled SD would estimate the method variation and the variability of the assay. As the measurements in this population are further away, we believe that the variability is due to the within subject variability. Moreover, none of our patients had

progressive disease as seen in the natural history graphs, so we believe that this is all due to natural variation. This was a retrospective observational study and the number of measurements varied from patient to patient. The interval between the measurements was not the same, as expected in a real-life setting. This limited the possibility of further analyses. The analysis for the within-subject SD did not take into account the effect of time. Ideally, to give a more accurate estimate of the variance, a prospective observational study should be designed, having the same number of measurements for each patient at regular intervals. The analysis should be preferably done using a model that takes time into account. Despite the limitations, this study provided a guide to how adjusted calcium varies between the different groups described.

The patients studied were identified from a referral centre, and they were evaluated for causes of secondary osteoporosis so that they might be different from the general population. On the other hand, NPHPT is usually diagnosed during the evaluation of secondary osteoporosis and thus, mainly found in referral centres. Studying a referral population has the advantage of evaluating how common NPHPT is in a referral population with osteoporosis and whether screening for NPHPT is useful.

Another group that is mentioned in this cohort, is the normoparathyroid hypercalcaemia one. These patients were previously been described to progress to primary hyperparathyroidism (Rejnmark et al, 2013). It was not within the scope of this study to evaluate these patients further, but it is amongst our future plans.

The question resulting from this study is whether NPHPT exists and, if it does, whether the current definition used is the most appropriate. We believe that there are three ways forward. If we adjust the reference interval for PTH for age, and use the current

definition, NPHPT may not exist, as shown in this study. Another approach would be to revisit the current definition and normocalcemic hyperparathyroidism should be defined as having the average calcium within normal limits (and not requiring it to be persistently normal). Using the same approach, PHPT should be considered as having a high average calcium. In this case, 10 out of our 11 included patients would be classified as NPHPT, and sixteen out of seventeen PHPT patients would be classified as having PHPT (Figure 3-5).

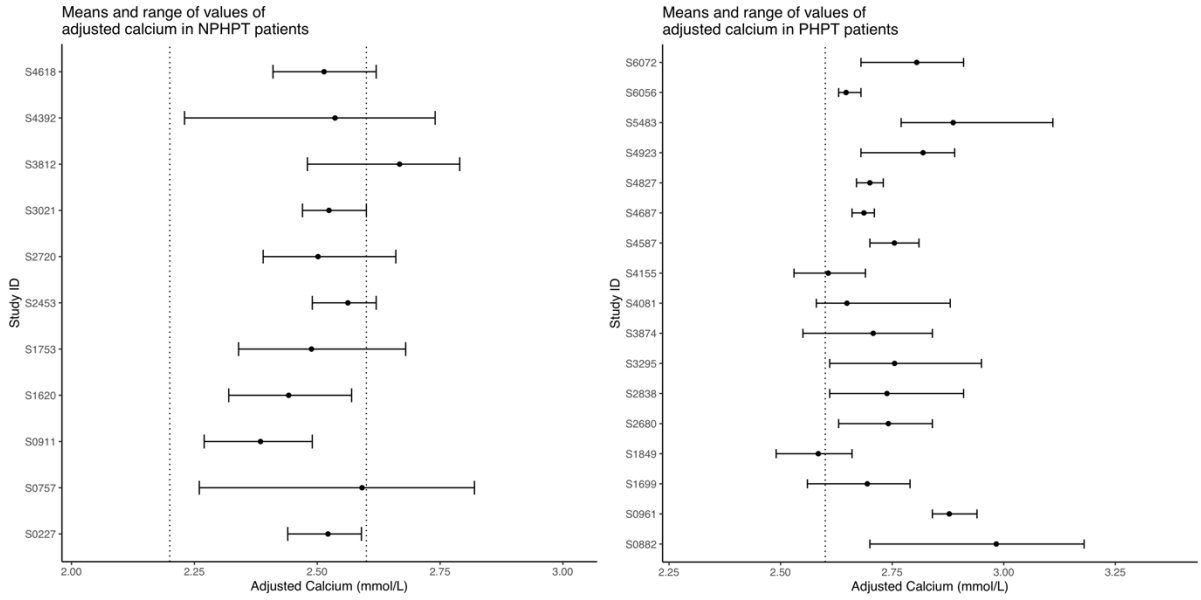


Figure 3-5: Means and range of values for adjusted calcium in the 11 NPHPT patients and the 17 PHPT patients.

The dashed lines represent the reference interval. This figure shows that if the mean calcium was used to define the different groups, all but one patients in the left graph would be classified as NPHPT and all but one patient from the right graph would be classified as PHPT. NPHPT: Normocalcaemic hyperparathyroidism; PHPT: primary hyperparathyroidism

Another way forward would be consider NPHPT as a variant of PHPT and follow up and treat according to the current guidelines as suggested in the consensus statement of the first European Society of Endocrinology Workshop (PARAT). This is supported by many studies in the literature showing similar characteristics from these two groups of patients. It is also supported by the fact that the underlying pathology of NPHPT seems to be similar to PHPT. We believe, based on the results from this study, that this is the best approach to follow in clinical practice.

In conclusion, persistent normocalcemia in patients with NPHPT is rare. If the international guidelines were strictly applied, the prevalence of NPHPT in this population would be zero. Our study is the first that studies the natural history of patients with NPHPT in so much detail and provides data from a long follow up period. We suggest that the definition should be revisited and normocalcemic hyperparathyroidism should be defined as having the average calcium within normal limits and not persistently normal. Subsequent hypercalcemia in these patients should be treated with caution, and any decisions for surgery should be made after persistently high levels of calcium have been confirmed.

Study	Population	Number of NPHPT patients (%); Mean age; Gender	Definition of NPHPT	Comments/limitations
Marques et al. **Brazil (referral centre)** **(Marques et al, 2011)**	Analysed records of 156 postmenopausal women who were referred to hospital to be screened for osteoporosis	14 (8.9); 60.6 years; 100% female	At least two samples of calcium and PTH Excluded: 25(OH)D<30ng/ml, GFR<40ml/min, medications (bisphosphonates, diuretics, anticonvulsants, lithium), metabolic bone diseases, gastrointestinal diseases with malabsorption, liver disease, incomplete records	Lower cut-off for eGFR
Šiprova et al. **Czech Republic (referral centre)** **(Siprova et al, 2016)**	Fifteen thousand three hundred forty-three referrals to Endocrine Centre. PTH measured in 1180 (patients with pathological or marginal levels of total calcium, ionised Ca, serum phosphate, patients with reduced BMD and with a possible PHPT diagnosis from medical history)	187 (1.2); 61.1 years; 81% female At follow-up 151 (81%)	Normal total and ionised calcium and high PTH at first visit 25(OH)D≥20ng/ml (patients with low vitamin D were treated, and PTH had to be elevated after retested at three months). Excluded cases with: renal insufficiency, calcium malabsorption, hypercalciuria, medications (PPI, thiazides, lithium)	Not clear if they excluded people on bisphosphonates, GFR cut off not given
Berger et al. **Canada** **(community)**	Population-based Canadian Multicentre Osteoporosis Study (CaMos): a prospective cohort of 9423 community-dwelling women	62 (3.31); NA NA	Normal adjusted calcium and high PTH, 25(OH)D≥50 nmol/L, eGFR≥60 ml/min/17.73m^2	85% were users of antiresorptives and diuretics. Not clear if they checked persistence or other causes

(Berger et al, 2015)	and men living within 50 km of nine Canadian cities. Included 566 men and 1306 women (n=1875) aged 35 years or older with available PTH			of secondary hyperparathyroidism
Cusano et al. **USA** **(community)** **(Cusano et al, 2013a)**	Dallas Heart Study (DHS): a population-based cohort study. Evaluated 3450 subjects aged 18-65 years with calcium and PTH values 2122 patients had follow-up data 8 years later	At baseline: 108 (3.1); 41.3 years; 38% female At follow up: 13 (0.6%)	Normal albumin-adjusted calcium and high PTH Excluded renal insufficiency (GFR<60ml/min), 25(OH)D≤20ng/ml, thiazide or lithium use	Only single laboratory values and did not check the persistence Lack of data regarding medical history and parathyroid surgery Not clear if they excluded patients on bisphosphonates or with hypercalciuria
Cusano et al. **USA** **(community)** **(Cusano et al, 2013a)**	Osteoporotic Fractures in Men (MrOS) study, an unselected community-based study in older men ≥65years. Evaluated 2364 men with calcium and PTH values	9 (0.4); 70.0 years; 0% female	Normal albumin-adjusted calcium and high PTH Excluded renal insufficiency (GFR<60ml/min), 25(OH)D≤20ng/ml, thiazide use	Same as DHS study
Garcia-Martin et al. **Spain** **(community)**	A prospective study in a cohort of 100 healthy postmenopausal women All the participants had follow-up six years later	6 (6); 56.3 years; 100% female	Normal adjusted calcium and high PTH 25(OH)D>30ng/ml, normal renal function (creatinine clearance>70ml/min/1.73m^2)	Not clear if they excluded patients on medications. Not clear what they defined as "healthy."

(Garcia-Martin et al, 2012)		At follow up: 6 (6%)		
Kontogeorgos et al. **Sweden** **(community)** **(Kontogeorgos et al, 2015)**	Random population sample of 2400 men and women aged 25-64 years from the World Health Organization MONItoring of trends and determinants for CArdiovascular disease (WHO MONICA) project. Investigation in 1995, data on 608 including all the women aged 45–64 years, every fourth woman aged 25–44 and every fourth man in all age groups (25– 64 years), n=410.	12 (2.0%); 53.3 years; NA At follow up: 1 (0.2%)	Normal total calcium and high PTH, 25(OH) ≥ 50 nmol/L, normal renal function	Patients on bisphosphonates, diuretics. Only one blood measurement at baseline
Lundgren et al. **Sweden** **(community)** **(Lundgren et al, 1997)**	Population-based mammography screening in 5202 women aged 55-75 years	28 (0.5) No data on age 100% female	Normal ionised calcium. Creatinine <160µmol/L and either a)calcium<2.50mmol/L+ PTH >55ng/l or b)calcium 2.50-2.60 mmol/L + PTH ≥35ng/l Checked for persistence (three or more occasions). Excluded malabsorption and family history of hypercalcemia	Did not exclude patients on medications known to cause secondary hyperparathyroidism. No vitamin D check
Palermo et al. **Five European cities in the UK, France, Germany (community)** **(Palermo et al, 2015)**	Recruited 2419 older women (55-79y) and 258 younger women (30-40y) for the Osteoporosis and Ultrasound Study (OPUS) Follow up available after six years in 1416 patients	1 (0.1); No information on age; 100% female	Mahalanobis distance used: NPHPT anyone outside the ellipse with normal adjusted calcium, high PTH, 25(OH)D≥50nmol.l, GFR≥60ml/min	Unclear if they excluded patients with other causes of secondary hyperparathyroidism (diseases, medication)

		At follow up: none		
Rosario et al. **Brazil** **(community)** **(Rosário & Calsolari, 2019)**	Prospectively recruited adults ≥18 years who would be submitted to thyroidectomy due to nodular disease. Excluded patients who had an ultrasound due to PHPT, patients with a history of nephrolithiasis, nephrocalcinosis and pathological fracture, personal or family history of multiple endocrine neoplasia or diagnosis of medullary thyroid cancer. N=676	Criterion 1: 46 (6.8%). Out of them only 8.7% had altered parathyroid glands (adenoma) during gland exploration (0.6% of the cohort) Criterion 2: 30 (4.4%). Confirmed pathology: 13.3% Criterion 3: 12 (1.8%). Confirmed pathology: 33.3% Criterion 4: 5 (0.74%).	Criterion 1: Normal adjusted and ionised calcium and high PTH, confirmed at two measurements, 25(OH)D ≥ 20 ng/dl, eGFR ≥ 40 ml/min/1.73 m^2. Excluded: people on diuretics, lithium, bisphosphonates, denosumab, recombinant PTH, corticosteroids; patients with primary aldosteronism, suspicion or known diagnosis of malabsorption, hyperphosphatemia, calcium/urinary creatine ratio ≥ 0.25, or thyroid dysfunction. Screened for coeliac disease and excluded patients with positive antibodies Criterion 2: same as criterion 1 but 25(OH)D ≥ 20 ng/dl, eGFR ≥ 60 ml/min/1.73 m^2 Criterion 3: same as criterion 1 but 25(OH)D ≥ 30 ng/dl, eGFR ≥ 40 ml/min/1.73 m^2 Criterion 4: same as criterion 1 but 25(OH)D ≥ 30 ng/dl, eGFR ≥ 60 ml/min/1.73 m^2	

		Confirmed pathology: 80%		
Vignali et al. **Italy** **(community) (Vignali et al, 2015)**	Residents of a village in Southern Italy in 2010 (685 with full data)	3 (0.4) 47 years 0% female	Normal adjusted calcium and high PTH, 25(OD) ≥30 ng/mL, eGFR ≥60ml/min/1.73m^2 Excluded people on bisphosphonates and thiazides, overt gastrointestinal and metabolic bone diseases	Did not check persistence and could not check urine calcium

Table 3-4: Studies evaluating the prevalence of normocalcemic hyperparathyroidism (NPHPT).

PTH: Parathyroid hormone; NA: not available

Study	Study population; duration of follow up	Definition of NPHPT	Progression to hypercalcemia
Ayturk et al. **(Ayturk et al, 2006)**	20; 18 months (6-month intervals)	Normal calcium and high PTH confirmed in at least three measurements. Excluded: chronic renal or liver failure, vitamin D deficiency, secondary hyperparathyroidism, treatment with lithium. No treatments with thiazide and loop diuretics, phenytoin, lithium, glucocorticoids, oral contraceptives during the study	None progressed to hypercalcemia
Tordjman et al. **(Tordjman et al, 2004)**	20; 4.1±3.2 years	Normal total calcium and high PTH Secondary hyperparathyroidism excluded (impaired renal function). Three patients had low vitamin D levels; however, correction did not alter PTH levels and did not unmask hypercalcemia. Six patients had >300mg/24h urine calcium, were given thiazides without affecting PTH levels Persistence not checked at baseline	None of the patients developed hypercalcemia. Their mean serum calcium levels did not change significantly (baseline versus last)
Garcia-Martin et al. **(Garcia-Martin et al, 2012)**	6; 1 year	Normal adjusted calcium and high PTH 25(OH)D>30ng/ml, normal renal function (creatinine clearance>70ml/min/1.73m^2) Persistence not checked at baseline	All the patients remained normocalcemic
Cusano et al. **(Cusano et al, 2013a)**	64; 8 years	Normal albumin-adjusted calcium and high PTH Excluded renal insufficiency (GFR<60ml/min), 25(OH)D≤20ng/ml, thiazide or lithium use Persistence not checked at baseline	Hypercalcemia: 1 (1.6%). Persistent normal calcium, high PTH: 13 (20%)
Diri et al. **(Diri et al, 2014)**	16; 4 years	Normal total calcium and high PTH, 25(OH)D >20ng/ml, repeated Ca and PTH measurements three times with 2-week intervals, no history of renal or liver diseases, no prescriptions known to affect calcium level	1 (6.25%) developed hypercalcemia

Kontogeorgos et al. **(Kontogeorgos et al, 2015)**	12; 13 years First assessment 1995, second in 2008-2009, participation rate 67%	Normal total calcium and high PTH, 25(OH) ≥ 50 nmol/L, normal renal function	1 (8.33%) developed hypercalcaemia Persistent normal calcium, high PTH: 1 (8.33%) Two had vitamin D deficiency, normal calcium and high PTH
Silverberg et al. **(Silverberg & Bilezikian, 2003)**	22; up to 1 year	Normal adjusted calcium and high PTH Confirmed on at least two occasions, eight patients had normal iCa, 25(OH)D>20ng/ml. Excluded FHH, liver disease, renal disease, urinary calcium>87.5mmol/24h, GI disease with malabsorption, metabolic bone disease, medications (lithium, thiazide, oestrogens, loop diuretics, bisphosphonates, anticonvulsants)	3 (14%) developed hypercalcemia
Siprova et al. (Siprova et al, 2016)	187; 1-7 years	Normal total and ionised calcium and high PTH 25(OH)D≥20ng/ml (patients with low vitamin D were treated, and PTH had to be elevated after retested at three months). Excluded cases with: renal insufficiency, calcium malabsorption, hypercalciuria, medications (PPI, thiazides, lithium)	151 (81%) remained normocalcemic for the whole follow-up period 36 (19%) became hypercalcemic with 13 (36%) being intermittently hypercalcemic
Lowe et al. (Lowe et al, 2007)	37; 3.1±0.3 years	Normal adjusted calcium and high PTH, 25(OH)D ≥50 nmol/L Excluded cases with renal insufficiency (GFR< 40 ml/min per 1.73 $m^{2)}$, liver disease; significant hypercalciuria >350mg/24h, thiazide diuretic or lithium use, other metabolic bone diseases (e.g. Paget's disease)	Seven (19%) became hypercalcemic. Patients who became hypercalcaemic had higher

			calcium levels, had higher urinary calcium excretion and were older

Table 3-5: Studies evaluating the natural history of normocalcemic hyperparathyroidism (NPHPT).

PPI: proton pump inhibitors; PTH parathyroid hormone

Section 2: Normocalcaemic hypoparathyroidism

Contribution details

Title	The prevalence and natural history of normocalcaemic hypoparathyroidism in a United Kingdom referral population
Publication status	The material presented in the following section has been written and formatted for submission in Clinical Endocrinology
Authors	Authors: Marian Schini[1], Rebecca Stirling[1], Richard Jacques[2], Eleanor Oakes[3], Nicola Peel[3], Jennifer Walsh[1], Richard Eastell[1] [1]Department of Oncology and Metabolism, University of Sheffield, UK [2]School of Health and Related Research (ScHARR), University of Sheffield, UK [3]Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT), UK
Authors roles	Study design: MS, RE. Data collection: MS, with support from RS (helped with some laboratory data extraction from ICE which MS then went through, corrected and updated as necessary). Data analysis: MS with advice from RJ. Interpretations: all authors. Drafting manuscript: MS. Revising manuscript: all authors. Approval of final manuscript: all authors
Student author's name	Marian Schini Signature

Co-author statement

I hereby declare that I am aware that the work in the manuscript entitled: "The prevalence and natural history of normocalcaemic hypoparathyroidism in a United Kingdom referral population" of which I am a co-author, will for part of the PhD dissertation by the PhD student Marian Schini who made a major contribution to the work stated above.

Co-author name
Rebecca Stirling
Richard Jacques
Eleanor Oakes
Nicola Peel
Jennifer Walsh
Richard Eastell

Supervisor confirmation

I have sighted email or other correspondence from all co-authors confirming their certifying authorship.

Name	Signature
Professor Richard Eastell	R Eastell 21/12/19

THE PREVALENCE AND NATURAL HISTORY OF NORMOCALCAEMIC HYPOPARATHYROIDISM IN A UNITED KINGDOM REFERRAL POPULATION

Short title: NHYPO: Prevalence and natural history

Authors: Marian Schini[1], Rebecca Stirling[1], Richard Jacques[2], Eleanor Oakes[3], Nicola Peel[3], Jennifer Walsh[1], Richard Eastell[1]

[1]Department of Oncology and Metabolism, University of Sheffield, UK

[2]School of Health and Related Research (ScHARR), University of Sheffield, UK

[3]Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT), UK

3.2.1 Summary

Context: Normocalcaemic hypoparathyroidism (NHYPO) is characterised by persistently low levels of parathyroid hormone (PTH) with normal levels of calcium. There is little in current literature on this disease, with only two studies published on its prevalence whilst its natural history remains relatively unknown.

Objectives: to identify the prevalence of NHYPO in a UK referral population and to study the natural history of the disorder.

Design: Retrospective study. Follow up five years

Patients: 6280 patients referred for a BMD measurement in a Metabolic Bone referral centre

Measurements: Prevalence of NHYPO and variability of calcium

Results: Based on laboratory results on the index day, 22 patients with NHYPO were identified. Four patients were excluded due to non-PTH induced hypocalcaemia and unconfirmed data. The final prevalence was 0.29%. Only 67% had persistent

normocalcaemia. Two of these patients also had persistently low PTH on two occasions. Most of the patients had one PTH measurement available. No patient developed permanent hypoparathyroidism.

Conclusions: The prevalence calculated from this UK referral population is lower when compared to results from previous studies. NHYPO patients often have episodes of hypocalcaemia with some cases having no apparent reason for calcium levels below the reference range.

Keywords: parathyroid diseases; hypoparathyroidism; hypocalcemia; epidemiology; prevalence; calcium metabolism disorders; parathyroid gland; calcium

3.2.2 Introduction

Calcium is an important mineral in the human body; its regulation within tight normal limits is of great importance. Parathyroid hormone (PTH) is the main regulator of calcium homeostasis and it regulates calcium levels by altering bone resorption, renal calcium excretion and the production of $1,25(OH)_2D$ (Favus & Goltzman, 2013).

Endocrine disorders are often characterised by a clinical and a subclinical form, such as subclinical hyperthyroidism, characterised by suppressed TSH with normal thyroid hormone levels (Biondi et al, 2015). Following a similar pattern, a new phenotype of PHPT has been presented recently in literature. Normocalcaemic hyperparathyroidism (NPHPT) is a disorder of calcium metabolism which is characterised by persistently normal calcium levels and consistently elevated PTH values after other causes of secondary hyperparathyroidism are excluded (Eastell et al, 2014).

On the other side of the spectrum of calcium related disorders, hypoparathyroidism is a rare disorder, characterised by low levels of calcium due to a low PTH secretion. The most common cause is acquired hypoparathyroidism, mainly seen after anterior neck surgery resulting in the damage of the parathyroid glands. A pathophysiological counterpart to NPHPT is normocalcaemic hypoparathyroidism (NHYPO), which is characterised by normal levels of calcium with persistent low levels of parathyroid hormone (PTH). To our knowledge, there is currently no official definition for this disorder. There is little in current literature on this disorder, with only two studies (reporting three different populations) published on its prevalence, whilst its natural history remains relatively unknown (Cusano et al, 2013a; Palermo et al, 2015). The prevalence in these studies is reported to be 1.1-2.4% at baseline. Two of these three populations which were quite different, [women from five European cities for the Osteoporosis and Ultrasound Study (OPUS) and participants of the Dallas Heart Study (DHS), a population-based cohort for the evaluation of cardiovascular disease] were then checked six and eight years later (1416 and 2122 patients respectively from each study went into follow up) and only 1.1% (out of the original 57 patients, 35 had blood measurements and only 15 had persistent findings) and 0.09% (out of the 68 patients identified, 26 had a follow up and 2 had persistent findings) respectively were still characterised as having NHYPO. None developed hypocalcaemic hypoparathyroidism. Serum calcium and PTH was only checked on two occasions in these studies, so the natural history of serum calcium is unclear. To our knowledge, no one has evaluated this before.

The primary aims of this study were to identify the prevalence of NHYPO and to study the natural history of the disorder, hypothesising that individuals presenting as NHYPO may have more labile calcium than the general population. A second aim was to compare the variability of adjusted calcium between NHYPO patients and a control group from the same cohort.

3.2.3 Materials and methods

3.2.3.1 Study population

The work was performed at the Metabolic Bone Centre (MBC) at Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT) in the United Kingdom. Data from patients referred for a bone mineral density (BMD) measurement between the 14th January 2013 to the 27th July 2017 were retrospectively evaluated. All the patients included in the study had a laboratory evaluation including calcium and PTH, performed within 28 days from their scan. In this department, a laboratory work-up for secondary osteoporosis is performed in patients having any of the following findings: low BMD for age (Z-score<-2), vertebral fracture and/or unexplained, accelerated bone loss since a previous scan. The day of the laboratory investigations was defined to be the index day. Any other results of calcium and PTH before and after the index day were retrieved from the hospital's records and were used to study the natural history of the disease. Although biochemical data were available from 2009 onwards, only the ones performed after the 14th January 2013 were used, because at that point the laboratory changed the assay for serum calcium and this would probably affect the accuracy of the results. The end of the follow up period was the 31st July 2018.

The analysis did not require any ethical approval according to the Sheffield Teaching Hospitals Clinical Research Office; it falls under the "case note review" category.

3.2.3.2 Biochemical measurements

Blood was drawn at any point of the day, so patients were not fasting. All the samples were analysed in the Chemical Pathology Laboratory, Sheffield Teaching Hospitals NHS

Foundation Trust. Serum calcium was measured using a Roche/Hitachi Cobas 8000 e702 automated clinical chemistry analyser (Roche Diagnostics GmbH, Mannheim, Germany). The interassay coefficient of variation as measured in the laboratory is 1.1 – 1.5% at 1.52 mmol/L and 0.6 – 1.1% at 3.07 mmol/L. Albumin measurement was performed using a Roche/Hitachi Cobas 8000 e702 analyser (Roche Diagnostics GmbH, Mannheim, Germany). The interassay coefficient of variation as measured by the laboratory is 1.5 – 2.4% at 33.9 g/L and 1.0 – 1.7% at 59.7 g/L. The albumin-adjusted calcium was used for this study and was calculated using the following equation (as used by the laboratory).

$$Adjusted\ Ca = Total\ Ca + [0.0172(43 - Albumin)]$$

The Pathology Harmony reference range has been in use for adjusted calcium since 2011; the range reported was 2.20-2.60 nmol/L (8.8-10.4 mg/dL) (Berg, 2014).

Intact PTH (second generation) was measured using an immunoassay method by the Roche Cobas 8000 e602 (Roche Diagnostics GmbH, Mannheim, Germany). The interassay coefficient of variation (CV) measured in the laboratory is 2.2 – 3.2% at 34 ng/L, 1.6 – 1.7% at 94 ng/L and 1.4 – 1.8% at 839 ng/L, while the reported reference range by the manufacturer was 15-65 ng/L (1.6 - 6.9 pmol/L).

25(OH)D was measured using a competitive binding protein assay and was performed by Roche modular analytics Cobas E170 (Roche Diagnostics GmbH, Mannheim, Germany). The laboratory interassay coefficient of variation for this assay is 6.5 – 9.9% at 48.2 nmol/L and 4.5 – 6.3% at 92.3 nmol/L. Patients with a level greater or equal to 50 nmol/L were considered replete (Aspray et al, 2014).

Creatinine was measured using a Roche/Hitachi Cobas c8000 e702 analyser (Roche Diagnostics GmbH, Mannheim, Germany). The interassay coefficient of variation for this

assay was 2.7 – 6.1% at 55.7 µmol/L and 2.3 – 4.0% at 458 µmol/L. The equation used to calculate eGFR was the Modification of Diet in Renal Disease (MDRD) Study equation:

$$eGFR\left(\frac{mL}{min \times 1.73\ m^2}\right) = 175 \times (\text{Serum creatine})^{-1154} \times age^{-0.203} \times [0.742\ if\ female] \times [1.212\ if\ black]$$

This equation changed in August 2015; the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation has been in use since then.

$$eGFR\left(\frac{mL}{min \times 1.73\ m^2}\right) = 141 \times \min\left(\frac{Scr}{k}, 1\right)^{\alpha} \times \max\left(\frac{Scr}{k}, 1\right)^{-1.209} \times 0.993^{age} \times 1.018\ [if\ female] \times 1.159\ [if\ black]$$

Scr is serum creatinine in mg/dL, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, max indicates the maximum of Scr/κ or 1 (KDIGO, 2012). A level ≥60 ml/min/1.73m^2 was considered as normal.

Alkaline phosphatase was measured using a Roche/Hitachi Cobas c8000 e702 analyser. The interassay coefficient of variation for this assay was 2.4% at 92.8 IU/L and 1.7% at 224 IU/L. The reported reference range was 30-130 IU. The same analyser was used for the measurement of serum phosphate and the interassay coefficient was 1.4% at 1.23 mmol/L and 1.2% at 2.04 mmol/L. The reported reference range was 0.8-1.5 mmol/L.

Bone mineral density was performed using dual-energy X-ray absorptiometry (DXA) of the lumbar spine and the proximal femur in posteroanterior projection. At the time of the study, there were three Hologic DXA scanners within the centre. The least significant change (LSC) used in the MBC was 4.5% for both the spine and the hip. There were three scanning rooms in the department and each room has a different scanner. Room A: Hologic Discovery A in use since 2010. Room B: Hologic QDR4500A was in use from 2011 until the 11th September 2013. The scanner was then decommissioned and replaced by a Hologic Discovery SL. Room C: Hologic Delphi C was in use until the 3rd November 2014. The scanner was then

decommissioned and replaced by a Hologic Horizon A. The different DXA scanners were cross-calibrated upon installation.

3.2.3.3 Statistical analysis

Due to the fact that calcium and PTH are not independent variables, it was considered best to use a bivariate statistical approach to define the different categories of patients. Mahalanobis distance is a multi-dimensional generalization of the idea of measuring how many standard deviations (SD) away an observation is from the mean of a distribution. It is a classical method of defining outliers in a multivariate point cloud and is defined for each point x_i, by the following equation, where MD_i: Mahalanobis distance, μ: arithmetic mean of the dataset and S: sample covariance matrix (this is a robust covariance matrix using the minimum volume ellipsoid method).

$$MD_i^2 = (x_i - \mu)^T S^{-1} (x_i - \mu)$$

The distance MD_i gives the distance of point x_i from the centre of the cluster of points, taking into account the shape of the cluster. Observations are considered outliers if $MD^2 > X^2_{2;0.975} = 7.378$ [97,5th percentile of a chi-squared (X) distribution with two degrees of freedom] (Rousseeuw & van Zomeren, 1990; Van Aelst & Rousseeuw, 2009). The correlation analysis of adjusted calcium and PTH was performed based on a log10 transformation of the two variables. A similar approach has been used in the past to evaluate the prevalence of NHYPO in a different group of patients (Palermo et al, 2015). Using this method, subjects were identified as “normal” if they were inside the ellipse and “abnormal” if they were outside. Using the laboratory reference intervals for calcium and PTH, patients were divided into 10 categories as described below. “High” or “low” are referred to values of either adjusted calcium or PTH being above or below the reference range respectively.

To compare the variability of calcium in the two groups, the within-subject standard deviation was calculated. The analysis of variance method was used to calculate the within subject variance, as this method deals with the case of subjects having different numbers of observations. To follow this method, we first checked the assumption that the standard deviation was unrelated to the magnitude of the measurement. This was done graphically, by plotting the individual subjects' standard deviations against their means and analytically by calculating a rank correlation coefficient (Bland & Altman, 1996a).

The independent samples t-test and the Mann-Whitney test were used to compare quantitative data, while chi-square was used for the analysis of categorical data. The results are presented as mean and standard deviation when the data were normally distributed, as geometric mean and 95% confidence intervals when log transformation was performed in order to achieve a normal distribution of the data, and median and interquartile range (IQR) in the case where non-parametric tests were used for the analysis. For the comparison of the overall measurements of PTH and adjusted calcium resulting from follow up, we used a mixed linear model. Individual variability was modelled using a random effect and the difference between groups was estimated using a fixed effect. The statistical analyses have been performed using the R studio statistical software version 1.1.442 (RStudio, Inc. Boston).

3.2.3.4 Definitions of the different groups

As mentioned in the statistics section, patients were divided into ten categories based on their laboratory intervals for adjusted calcium and PTH. The groups of interest for this study were defined as follows. Normal: anyone inside the ellipse; Provisional normocalcaemic hypoparathyroidism (NHYPO): normal adjusted calcium and low PTH on the index date.

Hypoparathyroidism: low adjusted calcium and low PTH. In order to characterise these groups further, the following procedures were followed.

For the NHYPO group, the patients' medical notes were reviewed to look for other causes of the abnormalities found. Any unconfirmed data were excluded. For the control population, a random sample of 300 subjects from inside the ellipse having normal eGFR and being vitamin D replete (as defined above) on the index date was chosen.

3.2.4 Results

In total, 6293 patients attended the Metabolic Bone Centre and were assessed for secondary osteoporosis from January 2013 to July 2017. All these patients had a BMD measurement and a laboratory evaluation. Thirteen patients did not have PTH available on the index day and were excluded. The total number of patients analysed was 6280; their mean age was 66 years (range 16-100) and 72% were female. All these patients were given a study ID number starting from S0001 to S6280. After applying the Mahalanobis distance, the ellipse seen on Figure 3-6 was formed by using the measurements of PTH and adjusted calcium from the index date (single measurement of each variable per patient).. In total, 5574 patients were inside the ellipse and were considered as "normal" while the rest were outliers. Twenty-two patients fulfilled the criteria for NHYPO. The evaluation of their medical files excluded four patients. Three patients were excluded because of non-PTH induced hypercalcaemia; S0051 due to myeloma, while S1743 and S5588 due to metastatic cancer. S0756 was excluded because of the results extracted by the IT department could not be confirmed.

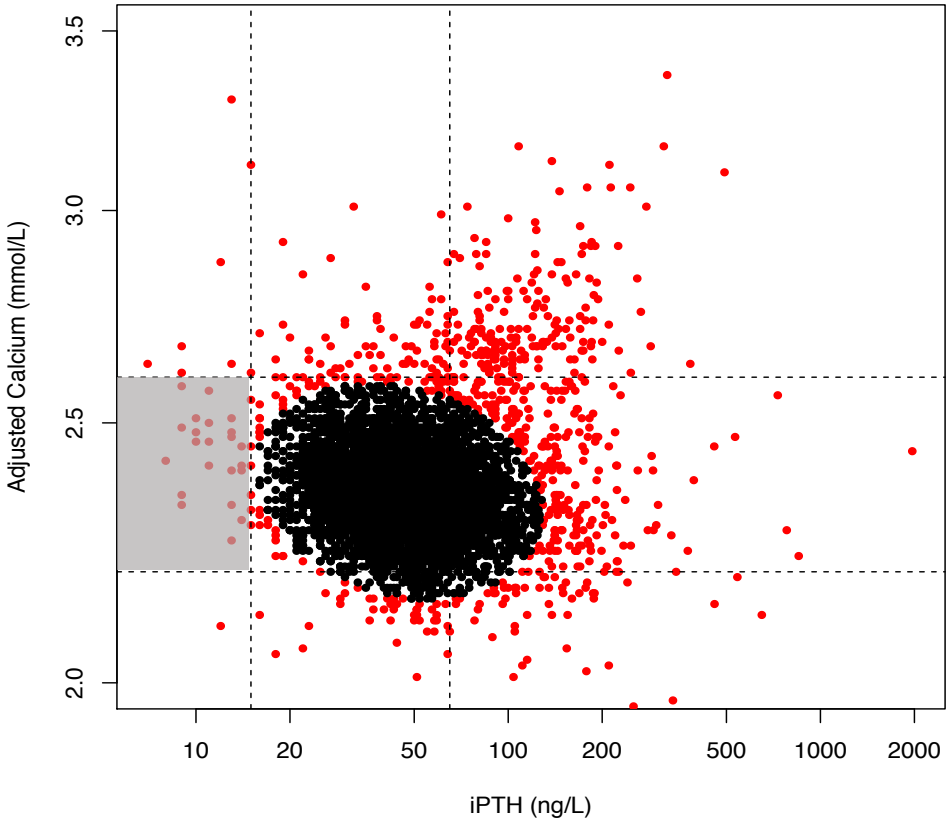


Figure 3-6: Data results from adjusted calcium and PTH.

The ellipse was formed using a statistical method (Mahalanobis distance) to identify "normal" subjects (black dots) and "abnormal" ones (red dots). The laboratory's reference range of both adjusted calcium and PTH (horizontal and vertical dashed lines respectively), were used to identify patient categories. The shaded area includes patients with normocalcaemic hypoparathyroidism (NHYPO).

The group of provisional NHYPO patients consisted of eighteen patients (mean age 45, SD 16.4 years, 61% female). The prevalence for this population was 0.29% (95% confidence interval 0.18-0.45%). None of these patients had previous anterior neck surgery or radiation. None of them was on active vitamin D. We noted that some patients had other conditions that could affect PTH results [thiazides n=1, antiepileptic medications n=4 (both conditions

can increase PTH levels); one patient had coeliac disease which can cause secondary hyperparathyroidism and thalassaemia which can cause functional hypoparathyroidism; four patients were consuming increased levels of alcohol)..

When studying the natural history, two patterns were identified (Figure 3-7): persistent normocalcaemia (in twelve patients, 67%) and intermittent hypocalcaemia (in five patients 28%). One patient only had one measurement of calcium during the follow up period (S5738). The minimum, maximum and average values of the adjusted calcium were calculated for each patient, and the graphs of the means and the range of values were drawn (Figure 3-8). There were no statistical differences in these two groups in terms of age, gender distribution, average adjusted calcium and PTH levels (all p values >0.05). In three of the patients with intermittent hypocalcaemia, the observed decreases in calcium could be explained (the most common cause was vomiting). One patient had one low measurement of calcium with no apparent reason explaining the results. Finally, one patient had one measurement of low calcium measurements during an epileptic seizure, one linked to low vitamin D levels and a third one where no obvious reason for hypocalcaemia was identified.

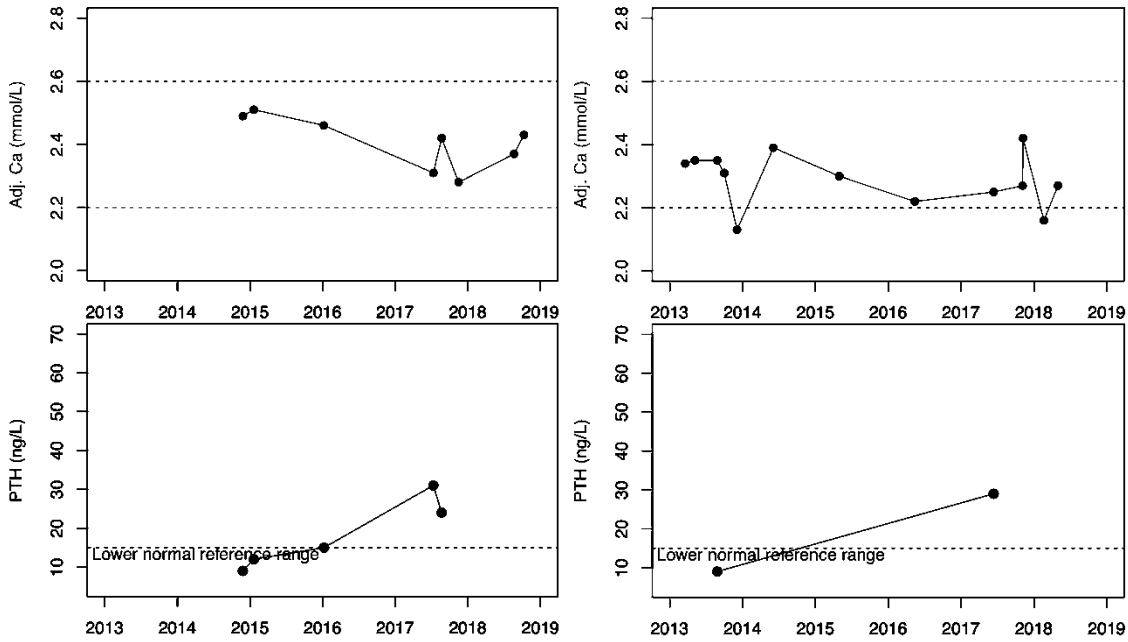


Figure 3-7: The two patterns identified in patients with normocalcaemic hypoparathyroidism when studying the natural history of adjusted calcium.

Persistent normocalcaemia (left top figure, patient S2157) and, intermittent hypocalcaemia (right top figure, patient S0161). The dotted lines represent the normal range for adjusted calcium. Both patients were on calcium and vitamin D supplements. The bottom graphs represent the variability of parathyroid hormone (PTH). None of the two patients maintained low parathyroid hormone throughout their follow up. The increase of PTH observed in the patient on the left, could be due to the initiation of zoledronic acid. The dotted line represents the lower normal of the reference range for PTH. Adj.Ca: adjusted calcium.

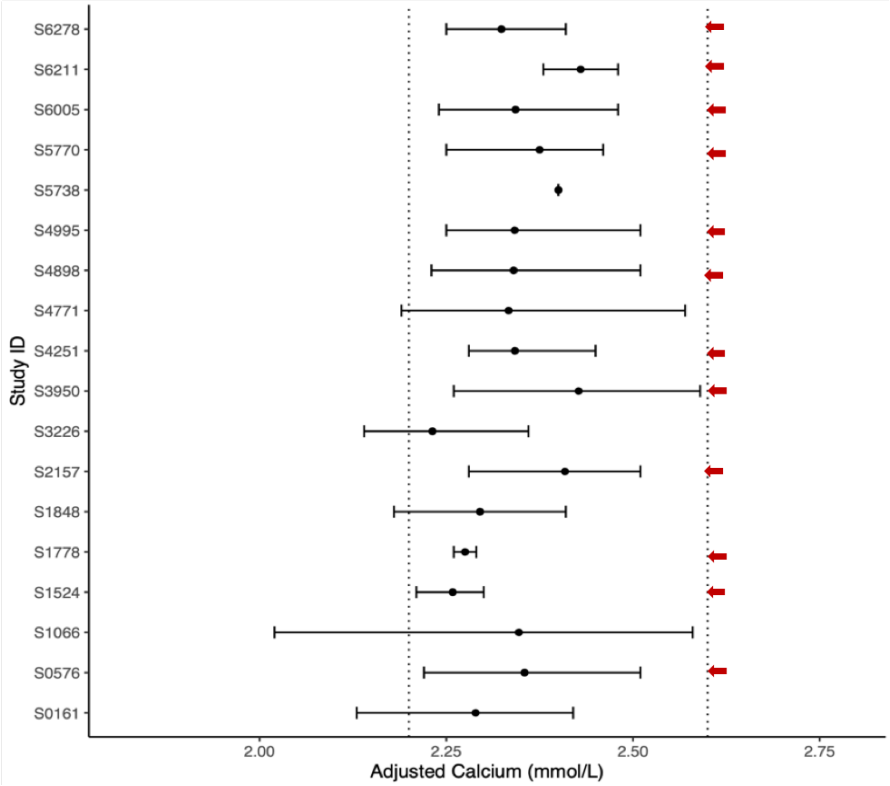


Figure 3-8: Means and range of values of different analytes in the 18 NHYPO patients.

Out of them, only 67% had persistent normocalcaemia (shown in the red arrows). One patient had only one measurement available. The dashed lines represent the reference range for adjusted calcium, NHYPO: Normocalcemic hypoparathyroidism

When looking at the number of available PTH measurements, eight patients had only one measurement of PTH during their follow up. Six patients had two measurements amongst which one was low, while one had both measurements below the reference range. Three patients had more than two measurements, with two of them having persistently low PTH on two occasions.

Only one patient from the whole population was found to have hypoparathyroidism. This patient had thyroidectomy for papillary cancer and developed hypocalcaemia as a result of this. The patient recovered spontaneously after a few months. The control population group

consisted of 300 subjects (mean age 67, SD 15.6 years, 71% female). The characteristics of the two groups on the index date are summarised in Table 3-6. There were statistically significant differences in the age and BMI of the two groups; NHYPO patients were younger and had lower BMI. The gender distribution did not differ between the groups. Patients with NHYPO had significantly lower PTH and higher phosphate. Alkaline phosphatase and vitamin D levels did not differ between the groups. The NHYPO group had lower age and gender adjusted BMD estimates.

	Control (n=300)	**NHYPO (n=18)**	**p value**
Female (%)	214 (71)	11 (61)	0.354
Age (years)[b]	70 (20)	47 (24)	**<0.001**
BMI (g/cm^2)[c]	25.6 (25.0 - 26.3)	22.6 (20.5 – 24.6)	**0.020**
PTH (ng/L)[c]	42.5 (40.8 – 44.1)	11.1 (10.3 - 11.9)	**<0.001**
Adjusted calcium (mmol/L)[a]	2.37 (0.08)	2.43 (0.09)	**0.006**
Phosphate (mmol/L)[a]	1.12 (0.18)	1.26 (0.18)	**0.003**
Alkaline phosphatase (IU/L)[b]	78 (37)	93 (36)	0.059
25(OH)D (nmol/L)[b]	78.9 (32.9)	78.3 (61.1)	0.561
Z score spine [a]	-0.2 (1.7)	-1.5 (1.2)	**<0.001**
Z score neck [a]	-0.4 (1.0)	-1.1 (1.0)	**0.011**

Table 3-6: Characteristics of the two groups on the index date.

[a] shown as mean (standard deviation); [b] shown as median (interquartile range); [c] results from multiple linear regression analysis after adjusting for age, weight and gender. The bold letters show the significant differences; BMI: body mass index; PTH: parathyroid hormone; NHYPO: normocalcemic hypoparathyroidism

Interestingly, the adjusted calcium on the index date was higher in the control population. However, after taking all the follow-up measurements into account, the mean adjusted calcium was found to be similar in the NHYPO group compared with the control group (mean adjusted 2.34 and 2.35 mmol/L for NHYPO and control group respectively, p=0.496). The variability of calcium in the NHYPO group was significantly higher than the control group [within-subject SD 0.096 (95% CI 0.085, 0.108) and 0.083 (95% CI 0.080, 0.085) mmol/L respectively). After taking all the follow-up measurements of PTH into account, the mean PTH was found to be lower in the NHYPO compared with the control group (mean PTH 15.8 and 48.0 ng/L respectively, p<0.001).

3.2.5 Discussion

Normocalcaemic hypoparathyroidism is a disorder characterised by low PTH and normal calcium on which limited information is available. To our knowledge, this is the first study of the prevalence in a tertiary centre and the also the first study of the natural history of this disease; previous studies only showed results from two time-points. We retrospectively evaluated 6280 patients attending a referral centre and identified eighteen patients with NHYPO on the index date (prevalence 0.29%). Out of them, only 67% had persistent normocalcaemia. The number of PTH measurements on these patients were limited, with only one patient having persistently low PTH.

The prevalence of NHYPO has been studied previously in the community. Palermo et al studied the prevalence in 2419 older (55-79 years old) and 258 younger (30-40 years old) women from five European cities for the Osteoporosis and Ultrasound Study (OPUS) and identified 57 subjects with NHYPO (prevalence 2.4%), using a similar approach as in our study. In order to get the different categories described above, they used the reference

range for adjusted calcium, but for PTH, they used the geometric mean of the reference range, which probably explains the higher baseline prevalence presented in their study (Palermo et al, 2015). We have adjusted this method and brought it closer to the everyday clinical practice, by using both the Mahalanobis distance and the reference range for both adjusted calcium and PTH. In the Palermo et al study, the adjusted calcium at baseline was similar to the control population, while PTH was significantly lower in the NHYPO group. Age, BMI and 25(OH)D were similar between the two groups. Six years later, the measurements were repeated once in 1416 subjects; only 0.6% of the initial population (1.1% of the ones that went into follow up) expressed persistent characteristics of NHYPO (Palermo et al, 2015). Our study showed some differences in baseline characteristics, with NHYPO patients being younger, having a lower BMI and higher adjusted calcium than the control. The age inclusion criteria in the OPUS study were more strict and thus the difference in the results. We only found a higher adjusted calcium at the index date. When taking all the repeated measurements into consideration, the levels between NHYPO and control were similar. The cohorts differed as OPUS participants were randomly selected compared to our cohort in whom there was clinical concern about bone health.

Cusano et al studied two non-referral populations, the Osteoporotic Fractures in Men (MrOS) and the Dallas Heart Study (DHS) (Cusano et al, 2013a). MrOS is an unselected community-based study in older men aged 65 years or older that had no bilateral hip fractures. They randomly selected 2503 subjects, and after excluding 139 without calcium measurements, they evaluated 2364 men from this study. Using the baseline values, they identified 26 participants (prevalence 1.1%) with NHYPO. They also found lower levels of PTH in the NHYPO group when compared to the group having normal PTH. The level of adjusted calcium was similar between the groups. They did not find a significant difference in age between the groups, unlike our study; however, they only included patients over 65

years. BMI was also similar between the groups, while in our study, NHYPO patients were slimmer. BMD results were similar (Cusano et al, 2013a). Our study found lower Z-scores in the NHYPO population on the index date. One explanation for this could be the slightly lower BMI in this group. Another possible explanation could be the fact that younger patients are more likely to have causes of secondary osteoporosis and thus relatively more affected than the older population investigated. Interestingly, this study found significantly higher consumption of calcium supplements ≥1000mg in the NHYPO group (54% versus 23% in the group with normal PTH). The dose of vitamin D supplements was similar between the groups (Cusano et al, 2013a). This could be the explanation to the fact that the adjusted calcium at baseline was slightly higher in the NHYPO group, an observation also seen in our study.

DHS is a population-based cohort for the evaluation of cardiovascular disease. In brief, this group evaluated 3557 subjects out of 6101 (aged 18 to 65 years) and excluded 107 because of lack of PTH or 25(OH)D levels, leaving 3450 subjects for the analysis (Cusano et al, 2013a). They reported a prevalence of 1.9% (68 patients were identified) from the baseline measurements. Age, BMI, baseline adjusted calcium and phosphorus did not differ between the groups. There was a statistical difference in vitamin D, with the patients with normal PTH having lower levels (Cusano et al, 2013a). This was not observed in our study, however we only included subjects with normal vitamin D at baseline in our control group. We also found higher levels of phosphate in the NHYPO group on the index date. The DHS study included a follow up (for 2122 subjects), where a single measurement was performed eight years later; out of the 26 subjects with NHYPO that went into follow up, none developed hypocalcaemia. Only two had persistent NHYPO (prevalence 0.09% of the population that went into follow up). Most of them (20 out of 26) had normal PTH (Cusano et al, 2013a).

Normocalcemic hypoparathyroidism was previously characterised as a state of "low bone turnover", as patients with this disorder were found to have lower bone ALP, collagen type 1 cross-linked C-telopeptide (CTX) and osteocalcin compared to the normal population but without a significant BMD change over time (Palermo et al, 2015). The authors suggested a critical evaluation of potential treatment for osteoporosis in these patients, as medications like bisphosphonates can suppress bone turnover further and exacerbate adynamic bone disease (Palermo et al, 2015). Moreover, studies with teriparatide did not improve BMD in patients with hypoparathyroidism (Cusano et al, 2012). The question of which treatment to use in this group of patients becomes more relevant in our study, as all the patients were identified during a laboratory investigation for secondary osteoporosis.

There was a limited number of PTH measurements throughout the patients' follow up in our study; eight patients out of the eighteen identified as having NHYPO had only one measurement available. This suggests that physicians are not so concerned about finding a low level of PTH and they do not tend to follow this up further. However, this disorder could be the subclinical form of hypoparathyroidism. Moreover, these patients might be more prone to developing hypocalcaemia after they receive medications that can affect calcium levels (eg bisphosphonates, loop diuretics, denosumab) or after they develop common symptoms; in our population, three of the NHYPO patients had incidence of hypocalcaemia due to vomiting. Interestingly, some episodes of hypocalcaemia in our patients could not be explained. Therefore, the low levels could be theoretically attributed to the variability of calcium. A concern is that hypocalcaemia can be a cause of cardiac arrhythmias and epileptic seizures (Cooper & Gittoes, 2008). One of the patients from this population had low levels of calcium during an epileptic fit.

Interestingly, four of our patients were known to have epilepsy and were on treatment with antiepileptics which are known to cause an increase in PTH, however, a low level was found

(Pack, 2003). One patient was on thiazides and one patient had coeliac disease, both known causes of high PTH. The patient with coeliac disease also had thalassaemia, so PTH could had been low due to the iron load in the parathyroids, a known cause of hypoparathyroidism. Another four patients were consuming increased levels of alcohol, which has been reported to decrease the levels of PTH (Venkat et al, 2009). Unfortunately, magnesium was not available to check for hypomagnesaemia.

Further information on this disorder is needed before any official recommendations are made but we think that NHYPO should be mentioned in any future guidelines on hypoparathyroidism as it is not mentioned in the current ones (Bollerslev et al, 2015; Brandi et al, 2016); its pathophysiological counterpart, normocalcaemic hyperparathyroidism, is already part of international guidelines (Bilezikian et al, 2014). As mentioned before, there is no official definition for NHYPO, but we propose the following definition: normal calcium with low PTH should be confirmed on at least two occasions, but normocalcaemia does not have to be persistent as long as the average level of calcium is normal; episodes of hypocalcaemia can be part of the presentation. As for hypoparathyroidism, we propose that NHYPO includes both idiopathic and functional forms of hypoparathyroidism.

More research should be performed to further characterise this population, ideally a prospective, long-term study with repeated measurements of calcium and PTH. The patients identified should then be further characterised with more dedicated studies of calcium metabolism. Genetic studies would also be of interest. An explanation for this disorder could be that it is a mild form of activating CaSR mutation which results in the alteration of the set point for PTH. A mutation on the CaSR was reported in patients with normocalcaemic hyperparathyroidism(Díaz-Soto et al, 2016).

This study describes the largest population ever studied to identify provisional NHYPO and provides data during a long follow up period (five years). It is the first study that provides data on the natural history with adjusted calcium measurements available on more than two timepoints. There are several limitations in this study. The patients studied were identified from a referral centre and they were evaluated for causes of secondary osteoporosis, so they might be different than the general population. The samples were not fasting, a tourniquet was used to take blood, ionised calcium and magnesium were not available and there were not many repeated measurements of PTH. The PTH molecule is rapidly degraded and it was difficult to exclude pre-analytical variations for an inappropriate low PTH level. This was a retrospective observational study and the number of measurements varied from patient to patient. The persistence of the results could not be always confirmed. The interval between the measurements was not the same, as expected in a real-life setting. This limited the possibility of further analyses. The analysis for the within-subject SD did not consider the effect of time. Ideally, to give a more accurate estimate of the variance, a prospective observational study should be designed, having the same number of measurements for each patient at regular intervals. The analysis should be preferably done using a model that takes time into account. Despite the limitations, this study provided a guide to how adjusted calcium varies between the different groups described.

In summary, we retrospectively evaluated a referral population and found a low prevalence of patients with normocalcaemic hypoparathyroidism. The most common pattern was persistent normocalcaemia (67%), but there were patients that had intermittent hypocalcaemia. None developed persistent hypoparathyroidism. The clinical implication of this study is that if a patient has low PTH but normal serum calcium they should have regular follow-up as they could become hypocalcaemic at some point and can have clinical consequences like epilepsy. The findings of normal calcium and low PTH have to be

confirmed before a certain diagnosis is made. Further studies are needed to characterise this group further.

Section 3: Further analyses

3.3.1 Introduction

In the previous two sections the prevalence and natural history of normocalcaemic hyperparathyroidism and hypoparathyroidism were studied, using a statistical approach called Mahalanobis distance and forming an ellipse with the different groups of patients.

In order to check whether different characteristics of the population like age, vitamin D status and eGFR level would affect the ellipse, some further analyses were planned and presented here.

Moreover, in order to calculate the within-subject SD in these groups, I made some assumptions like non-progression of the disease and used an approach described in literature (Bland & Altman, 1996a) which might underestimate the variability for some patients (Masse, 1997). In order to try and overcome these limitations and supplement the previous results, I performed some further analyses. One approach suggested is to use the 90th centile of the within subject variance (Masse, 1997). I also looked whether these patients progressed through time and recalculated the within-subject SD after excluding these patients.

3.3.2 Results

3.3.2.1 The effect of various factors on the shape and position of the ellipse

3.3.2.1.1 Original ellipse: the patients selected

This ellipse (Figure 3-9) shows the different patients included in the analyses for normocalcaemic hyperparathyroidism (NPHPT). It was interesting to see that all the selected NPHPT were at the upper normal range of adjusted calcium.

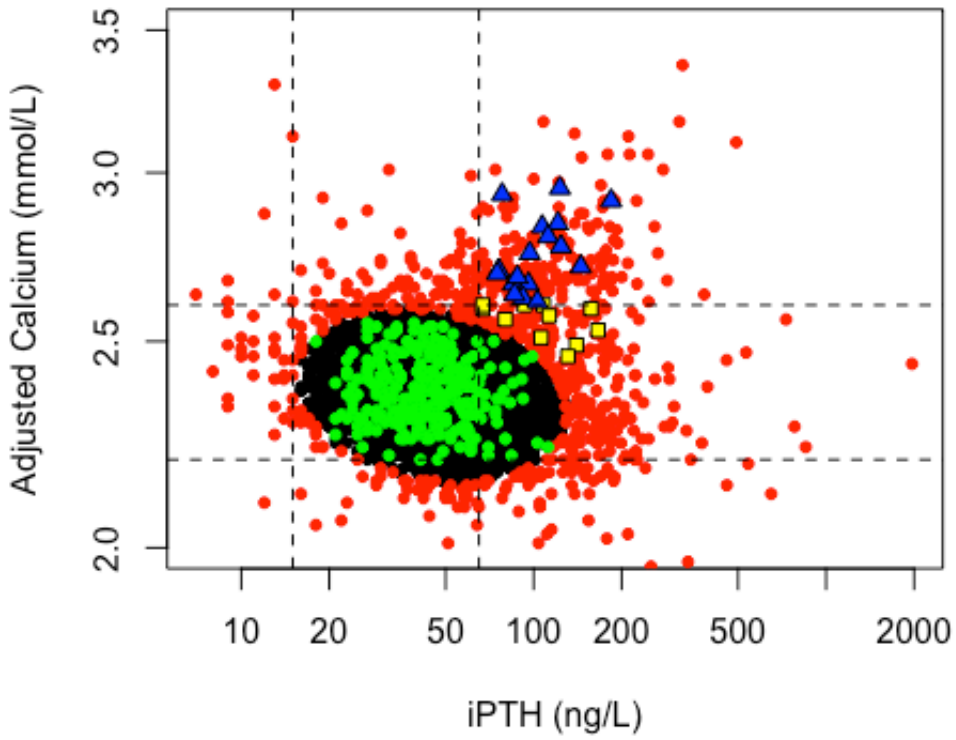


Figure 3-9. Ellipse showing the selected patients from the normocalcaemic hyperparathyroidism (yellow squares), primary hyperparathyroidism (blue triangles) and the control group (green circles)

3.3.2.1.2 Effect of age, kidney function and vitamin D status

In order to see how the format of the ellipse described in previous chapters changes with increasing age, CKD and vitamin D status, different ellipses were formed as seen in the following figures. The code for doing this can be found in the Supplementary material (code 4.2).

As expected, as age increases, the number of people with higher PTH increases and the ellipse moves to the right. Similar results were observed with vitamin D deficiency and CKD; increasing numbers of subjects had higher PTH levels.

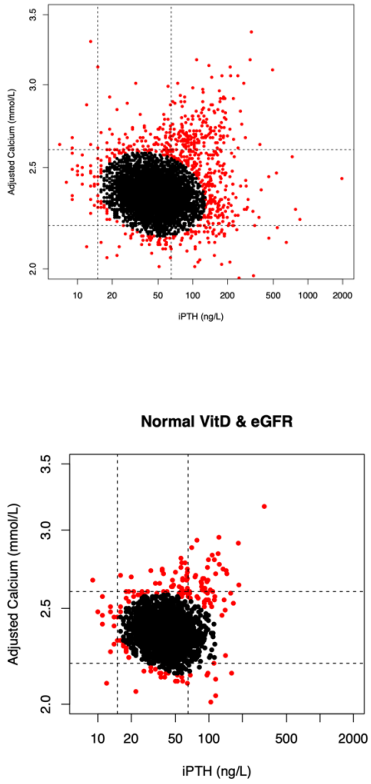


Figure 3-10: Comparison of the ellipse formed using all patient information (n=6280) and the one formed by subjects being vitamin D replete and having normal eGFR.

Fewer people have PTH levels which are outside the reference interval

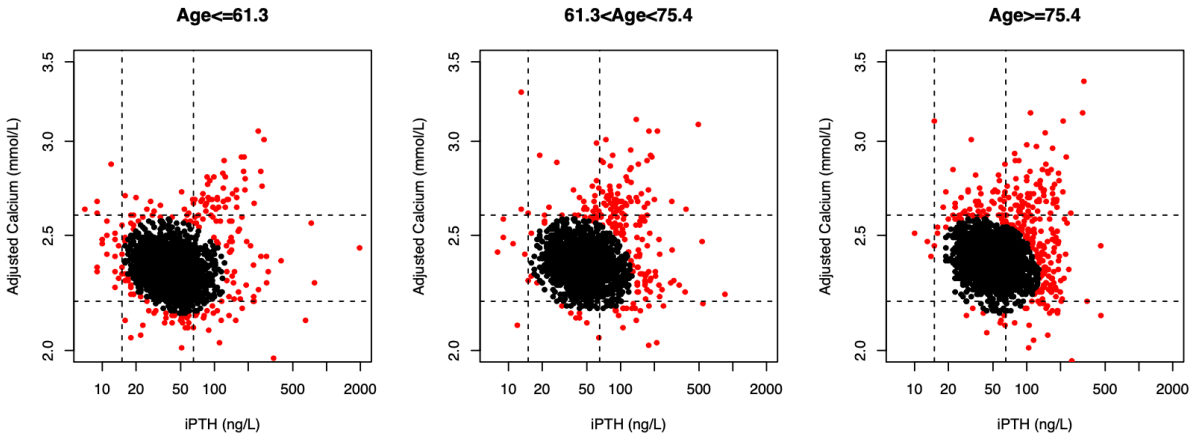


Figure 3-11: The effect of increasing age on the shape and position of the ellipse.

The patients were grouped in tertiles as follows. 1: age ≤61.3 years; 2: age 61.3 - 75.4 years; 3: age ≥ 75.4 years. As age increased, the ellipse moved towards the right and more people were found to have increased PTH, either presented as hypercalcaemic hypercalcaemia or isolated increased PTH with normal calcium

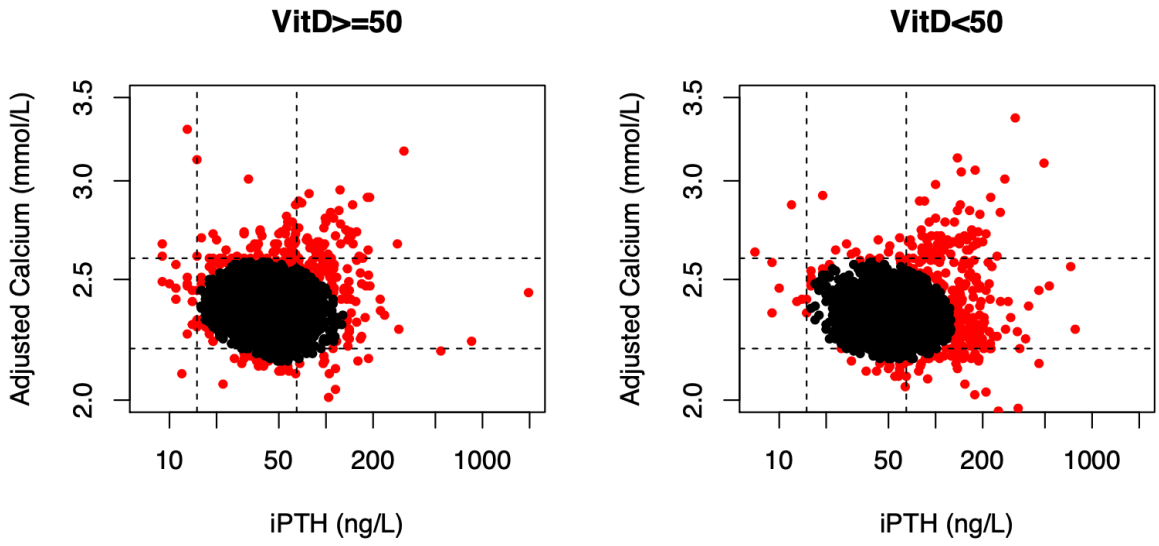
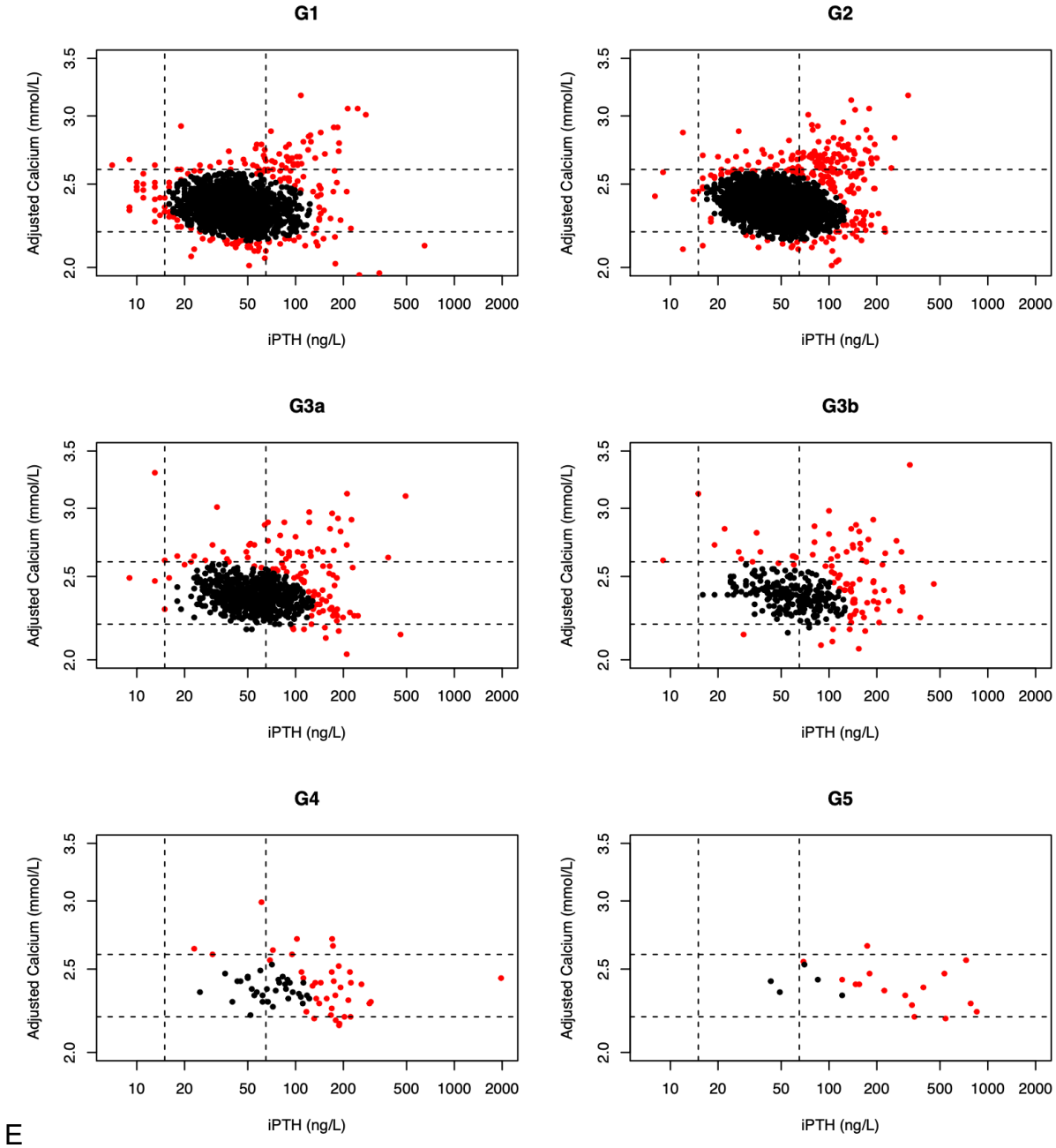


Figure 3-12: The effect of vitamin D status in nmol/L on the ellipse.

As expected, when evaluating vitamin D deficient subjects (on the right), more people were found to have high PTH with normal calcium (consistent with secondary hyperparathyroidism)



E

Figure 3-13: The effect of kidney function on the ellipse.

The different stages are defined as follows. G1: ≥90, G2: 60-89, G3a: eGFR 45-59, G3b: 30-44, G4: 15-29, G5: <15 ml/min/1.73 m^2 (KDIGO, 2012). As expected, patients with lower eGFR were more likely to have higher PTH values (consistent with secondary hyperparathyroidism)

3.3.2.1.3 Comparison of the subjects within the normal population

I used a statistical approach to define the different categories in this population. The results showed that a large percentage of subjects were considered "normal" (inside the ellipse) using this approach, even though they had elevated PTH according to the laboratory reference interval. The subjects that were inside the ellipse but had higher PTH than the upper limit of the reference interval (group 2), were found to be significantly older than the ones that were inside the ellipse and also had normal PTH according to the current reference interval (group 1) (Figure 3-14). Their mean age of group 1 and 2 was 64 and 71 years respectively ($p<0.001$). The mean vitamin D was lower in group 2 (45 vs 62 nmol/L in group 1, $p<0.001$). The mean eGFR was also lower in group 2 (68 vs 78 ml/min/1.73m^2 in group 1, $p<0.001$). These results align well with the fact that PTH is known to increase with age.

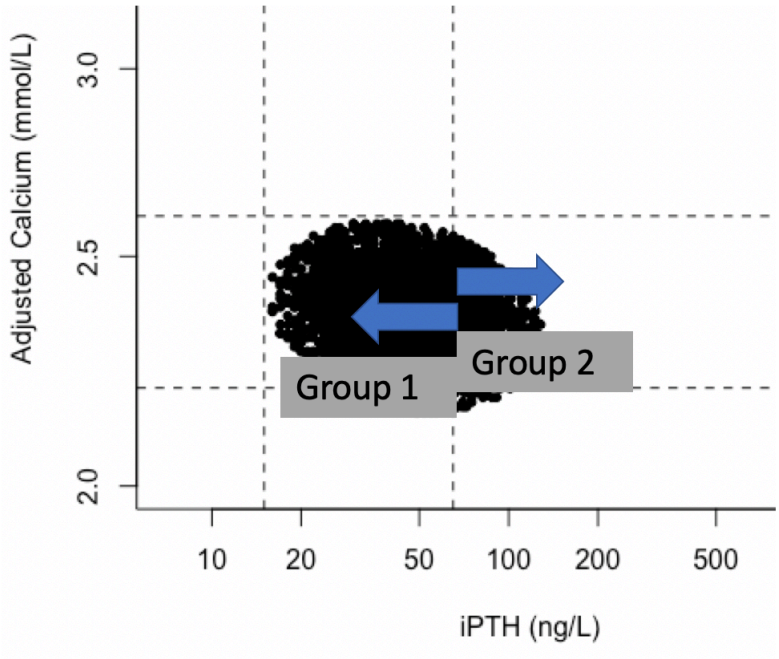


Figure 3-14: There were two groups of subjects inside the ellipse.

The subjects that were inside the ellipse but had higher PTH than the upper limit of the reference interval (group 2); the ones that were inside the ellipse and also had normal PTH according to the current reference interval (group 1)

3.3.2.1.4 Parathyroid hormone and age

When dividing the population in age tertiles (Q1: age ≤61.3 years; Q2: age 61.3 - 75.4 years; Q3: age ≥ 75.4 years), there was a significant difference in PTH in the three groups ($p<0.001$), with PTH being higher as age increased (Figure 3-15). The same results were found when repeating the analysis with only patients with normal eGFR and vitamin D.

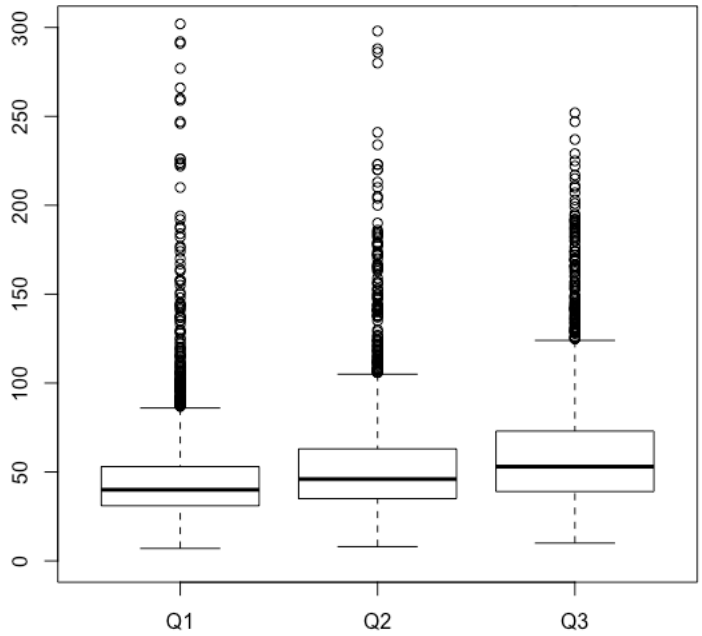


Figure 3-15: Boxplot of the different PTH levels according to age group

Q1: age ≤61.3 years; Q2: age 61.3 - 75.4 years; Q3: age ≥ 75.4 years; p<0.001

PTH was positively correlated with age. A multiple linear regression was then performed, adjusting for different factors (age, sex, vitamin D, eGFR, adjusted calcium, phosphate, BMI). Overall, age, eGFR, vitamin D, adjusted calcium and phosphate were independent predictors of the PTH level.

The multiple linear regression analysis was repeated after excluding patients with low vitamin D and eGFR, adjusting for the same factors as before. In this case, age, vitamin D, adjusted calcium, phosphate and BMI were independent predictors of the PTH level.

3.3.2.2 Within subject standard deviation analyses

3.3.2.2.1 Using the 90th centile

I used an approach described by Bland & Altman (Bland & Altman, 1996a) to calculate the within-subject standard deviation for adjusted calcium in the referral population.

In order to perform this analysis, the assumption that standard deviations across the subjects are similar should be made. However, this is not true in all clinical situations. Using a single "mean" within-standard deviation might underestimate the variability for some patients (Masse, 1997). I understand that this is a limitation of this approach and that the true within-subject SD might be bigger.

A suggested approach to overcome this problem is to use the 90th centile of the within subject variance. This method is considered to be a conservative approach (Masse, 1997).

The code to calculate these variables can be found in the Supplementary material (code 4.1). The results are seen in Table 3-7.

	Previously calculated SD (mmol/L) using analysis of variance	90th SD (mmol/L)
Control	0.083	0.105
NPHPT	0.089	0.115
NHYPO	0.096	0.163
PHPT	0.088	0.112

Table 3-7: Results for within-subject standard deviation (SD) for adjusted calcium when taking into account the 90th centile of the variances.

NPHPT: normocalcaemic hyperparathyroidism, NHYPO: normocalcaemic hypoparathyroidism, PHPT: primary hyperparathyroidism

3.3.2.2.2 Looking for disease progression and recalculating within subject SD

Another limitation of using the above mentioned approach when calculating the within-subject SD for the different categories of patients (NPHPT, NHYPO, PHPT, controls) is that I am making the assumption that all patients remain stable and there is no disease progression, therefore that variations are due to random variation. To check this assumption, I used the history graphs of adjusted calcium over time for every subject fitted with a regression line (n=11 NPHPT, n=17 PHPT, n=18 NHYPO, n=300 controls). I looked for subjects that had a significant trend, upwards or downwards ($p<0.05$).

Overall, one subject with NHYPO showed a downward trend (6%), two subjects with PHPT showed an upward trend (12%), two subjects with NPHPT had a downward trend and one had an upward trend (23%) and 37 controls had a trend (12%), with 19 being downward. As these percentages were greater that what would be expected from a type I error (probability of rejecting the null hypothesis given that it is true, 5% with p value of 0.05), I decided to recalculate the SD after excluding these subjects.

	Previously calculated SD (mmol/L) using analysis of variance	SD (mmol/L) after excluding subjects with trends
Control	0.083	0.083
NPHPT	0.089	0.083
NHYPO	0.096	0.099
PHPT	0.088	0.080

Table 3-8: Results for within-subject standard deviation (SD) for adjusted calcium after excluding subjects that had a upwards or downwards trend through time when plotting adjusted calcium against time.

NPHPT: normocalcaemic hyperparathyroidism, NHYPO: normocalcaemic hypoparathyroidism, PHPT: primary hyperparathyroidism

The recalculated SDs were slightly lower than the previous ones, as expected. The only exception was NHYPO. This could be due to the fact that the subjects had the greater variability.

When the Bonferroni correction was applied to these analyses, no patient had a significant trend.

3.3.2.2.3 Least significant change

The least significant change is a term used in everyday practice in order to evaluate whether one measurement has significant change from the previous one. This can be calculated for a 95% confidence level and is done by multiplying the within-subject SD by 2.77 as mentioned in the methods (Table 3-9).

The least significant change given, could be used in two ways in these patients. First, it gives a range to which values are expected. For example, for a patient with NPHPT having an adjusted Ca of 2.55 mmol/L, the LSC is 0.25, so the next measurement would lie between 2.30 and 2.80 mmol/L, hence it could be high. This can provide some reassurance for the patient and the physician. Moreover, it can be a marker of whether an individual has an actual significant change of his calcium. Some patients with NPHPT are being operated and using the given LSC estimates, the physician can make sure that there was a statistically significant drop in calcium as a result of the parathyroidectomy. In NHYPO patients, it can be a marker of whether an individual has an actual significant change of their calcium in case of observed hypocalcaemia after receiving a medication or after a disease that can cause low calcium.

According to the international guidelines, one of the recommendations for surgery on PHPT is when the serum calcium is more than 0.25 mmol/L from the upper limit of normal. The panel recommended this threshold based on two considerations, namely symptoms and variability (Bilezikian et al, 2014). It was interesting to see that the LSC in a patient with PHPT who does not have CKD or vitamin D deficiency at baseline in the MBC population was 0.24 mmol/L. Of course, if a patient fluctuates between deficiency and normal vitamin D status, this would have a larger LSC.

LSC (mmol/L)	Using the analysis of variance approach	Using the conservative approach	After excluding subjects with trends
Control	0.23	0.29	0.23
NPHPT	0.25	0.32	0.23
NHYPO	0.27	0.45	0.27
PHPT	0.24	0.31	0.22

Table 3-9: Least significant change for the different groups.

This was calculated using: the original data (analysis of variance approach); the results from the conservative approach of taking the 90th centile of the variances; the results after excluding subjects that had a upwards or downwards trend through time when plotting adjusted calcium against time. NPHPT: normocalcaemic hyperparathyroidism, NHYPO: normocalcaemic hypoparathyroidism, PHPT: primary hyperparathyroidism

Chapter 4: UK Biobank analyses

Section1: Study of calcium variability

Section 2: Comparing the UK Biobank to the Metabolic Bone Centre population

Section 3: Reference range for adjusted calcium

Section 1: UK Biobank studies

4.1.1 Introduction

In the previous chapters, the natural history of normocalcaemic hyperparathyroidism and hypoparathyroidism has been reported and it was found that persistent normocalcaemia in these disorders is rare and these patients often experience intermittent hypercalcaemia (NPHPT) and hypocalcaemia (NHYPO). Measures of the variability of adjusted calcium in the different groups were calculated and presented (NPHPT, NHYPO, controls, primary hyperparathyroidism).

This phenomenon complicates the management of these patients. However, the problem with these studies, was that they were performed in a tertiary referral population and the results may not be generalisable in the wider United Kingdom (UK) population.

In order to study the variability of adjusted calcium further, data from the general population were studied. UK Biobank is the largest health resource in the United Kingdom, with data on half a million participants aged 40-69 years, equally represented from both sexes.

4.1.2 Methods

4.1.2.1 Setting

In brief, I wanted to compare the results found from the MBC population projects, to data coming from the general population. I wanted to know how representative the MBC population was and whether the results could theoretically be generalised to the wider population.

I looked for already collected data from a UK population and the best-established database for these studies at the time of the study was the UK Biobank. This project was in collaboration with Dr Fadil Hannan in the University of Liverpool who kindly completed the applications for retrieving the data and supported the project.

All the information included in this section is available at the official UK Biobank website (https://www.ukbiobank.ac.uk) (UKBiobank).

4.1.2.2 The UK Biobank

UK Biobank is a large health resource which has the aim of improving prevention, diagnosis and treatment of a wide range of serious illnesses. It is a registered charity with an initial funding of approximately £62 million. It was established by the Wellcome Trust medical charity, Medical Research Council, Department of Health, Scottish Government and the Northwest Regional Development Agency. It also had funding from the Welsh Government, British Heart Foundation, Cancer Research UK and Diabetes UK. It is also supported by the National Health Service (NHS).

UK Biobank has data on half a million participants aged 40-69 years, equally represented from both sexes. These were recruited throughout the UK between 2006 and 2010. Apart from the baseline visit, a repeat assessment visit was also carried out between August 2012 and June 2013 at the UK Biobank co-ordinating centre in Stockport. This included a subset of 20000 participants who lived within a 35 km radius of the assessment centre.

There were twenty-two assessment centres throughout the UK (Figure 4-1). In Sheffield (ID 11014), patients were recruited between August 2009 and July 2010 (n=30399, 5.81% of the total population).

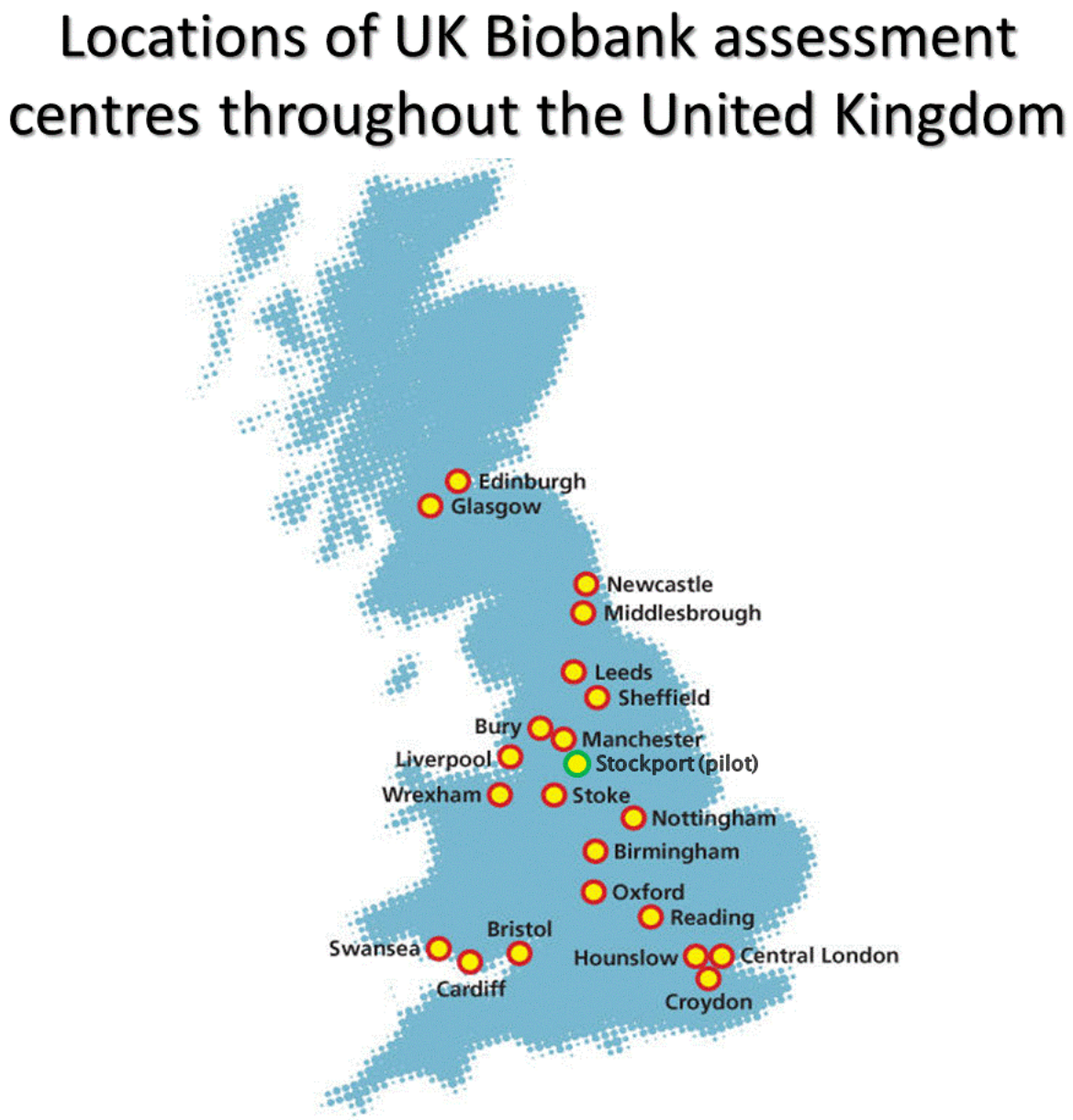


Figure 4-1: Map of assessment centres for the UK Biobank study.

Figure used with permission from the UK Biobank

4.1.2.2.1 Data included in the UK Biobank

The data available by the UK Biobank are divided in the categories described below. All the data are labelled in the format “F_I_A”, where F is the field ID, I is the instance index (see below) and A is the array index (if more than one measurement was performed on the same

date). An example is given for calcium: 30680-0.0 is the baseline assessment and 30680-1.0 is the first follow up. Only one measurement was performed on each day.

1. Data collected in the assessment phase (instance 0)

UK Biobank designed twenty-two special assessment centres throughout the UK to recruit patients. Data collected at the assessment visit included

- Information on a participant's health and lifestyle, hearing and cognitive function, collected through a touchscreen questionnaire and brief verbal interview
- Physical measurements, which included: blood pressure, arterial stiffness, eye measures, body composition measures, hand-grip strength, ultrasound bone densitometry, spirometry, and an exercise/fitness test
- Samples of blood, urine and saliva

There was a pilot phase of the recruitment performed in 2006 including over 3000 participants. There have been some differences in the data collected between the pilot phase and the main recruitment and differences could be seen in the data; pilot data fields have the identifier "pilot" in the name.

2. Data collected at the repeat assessment centre (instance 1)

As mentioned above, participants living within a 35 km radius of the assessment centre were invited to a further appointment. This included 20000 participants.

4.1.2.2.2 Timelines of data collection and release of data

Pilot phase

March - June 2006: UK Biobank Pilot Assessment

Main Phase (baseline assessment)

April 2007

- Opening of the first follow up centre (touchscreen questionnaire, physical measurements, biological samples)

Additional data collected

April 2009

- Touchscreen Questionnaire: Hearing test, cognitive function tests
- Heel ultrasound (both heels, previously just one), arterial stiffness
- 24-hour recall diet questionnaire

August 2009

- Eye measures (visual acuity, autorefraction, intraocular pressure)
- Blood collection: RNA blood sample, saliva sample

December 2009

- Eye measures (optical coherence tomography)
- Exercise test/ECG

July 2010: Recruitment ends

March 2012: UK Biobank Resource was launched

The data release from the UK Biobank was gradual.

4.1.2.2.3 Characteristics of responders versus non-responders in the repeat assessment

The information in this section was retrieved from the UK Biobank Document Repeat Assessment: Participant Characteristics of responders vs. non-responders, Version 1.1 July 2014 (UKBiobank).

As mentioned above, participants who lived within a 35 km radius from the repeat assessment centre were invited to participate in the repeat assessment in Stockport, between August 2012 and June 2013. In total, 103514 participants were invited to participate. Data was available on only 20345 (response rate 20%). There were no differences in gender (52.8% vs 51.1% female at baseline and repeat respectively), ethnic background (96.3% vs 97.6% white), alcohol drinker status or self-reporting of cancer or diabetes status at baseline. However, there were differences in the following characteristics.

The participants who agreed to attend for an assessment visit were more likely to be older (28% were between 60 and 65 years, Table 4-1). They were more likely to have a lower BMI (<25 kg/m^2), with 37.5% attending versus 31.6% invited to attend, to have no long-standing illness, disability or infirmity (68% of the ones that re-attended), to be of excellent health (20%), be “never smokers” and have higher education. Moreover, they were more likely to be less socioeconomically deprived and to live closer to the centre.

Age group at baseline	Baseline assessment (instance 0), %	Repeat assessment (instance 1), %
<45 years	10.5	7.7
45 to <50 years	13.6	10.7
50 to <55 years	15.6	14.6
55 to <60 years	18.8	22.3

60 to <65 years	24.4	28.2
≥65 years	17.1	16.6
Total	N= 103514	N=20345

Table 4-1: Age characteristics of the participants that were invited to participate in the repeat assessment and the ones that actually attended

4.1.2.2.4 Ethical issues in the UK Biobank

The UK Biobank funders developed a public Ethics and Governance Framework (EGF) to set the standards and the EGF went through a public consultation process. An independent Ethics and Governance Council (EGC) was formed in November 2004 to oversee UK Biobank's adherence to the Framework. UK Biobank had approval from the North West Multi-Centre Research Ethics Committee (MREC), covering the UK. Moreover, and to be able to gain access to information that would allow it to invite participants, the project had to be approved in England and Wales from the Patient Information Advisory Group (PIAG). PIAG had been replaced by the National Information Governance Board for Health & Social Care (NIGB). In Scotland, UK Biobank had approval from the Community Health Index Advisory Group (CHIAG).

4.1.2.3 Laboratory measurements available by the UK Biobank

The information in this section was retrieved by Document Ref: BCM023, Version 1.0, 12 Aug 2015, the Companion Document to Accompany Serum Biomarker Data, Version 1.0, 11 March 2019 and the Biomarker assay quality procedures: approaches used to minimise systematic and random errors (and the wider epidemiological implications), Version 1.2, 02

April 2019 (UKBiobank). Some further information was obtained after communication with the UK Biobank.

The biomarkers were measured in Stockport using the most up-to-date analytical methods that were available. At the time of this study there were 34 biomarkers available. These were (measured in serum unless stated otherwise):

- Cardiovascular: Cholesterol, direct low-density lipoprotein, HDL cholesterol, triglyceride, apolipoprotein A (APOA), apolipoprotein B (APOB), C reactive protein (CRP), lipoprotein A (LPA)
- Bone and joint: vitamin D, rheumatoid factor (RF), alkaline phosphatase, calcium
- Cancer: Sex hormone-binding globulin (SHBG), testosterone, oestradiol, insulin growth factor 1 (IGF-1)
- Diabetes: HbA1c (red blood cells), glucose
- Renal: cystatin, creatinine, total protein, urea, phosphate, urate, creatinine (enzymatic, measured in urine), sodium (urine), microalbumin (urine), potassium (urine)
- Liver: albumin, direct bilirubin, total bilirubin, gamma glutamyltransferase, alanine aminotransferase (ALT), aspartate aminotransferase (AST)

4.1.2.3.1.1 Sample collection and storage

The samples collected included blood (about 45 ml), urine (about 9 ml) and, for the last 100,000 participants, saliva. The participants were not fasting and venepuncture was performed using a tourniquet. The participants could have their blood measurements at any point in the day.

The pilot studies showed that several assays could be performed in samples maintained at 4°C for up to 36 hours prior to processing and storage. The only processing that was done immediately involved inverting the tubes, followed by a 30-minute rest to allow the serum tube to clot at room temp. Tubes were refrigerated until the end of the day when they were packed (with temperature logging devices) and transported to UK Biobank's central processing and archiving facility in Stockport. At the central laboratory, automated systems were used to generate 1.4mL aliquots (up to four aliquots from a participant sample). There was an average time of 24 ± 2.5 hours between venepuncture and sample storage.

Half of sample aliquots were stored in a fully automated -80°C working archive and half in a manual, nitrogen-vapour back-up archive located at separate sites in order to protect them.

In order to ensure that samples from the same geography and same dates and times were not picked up and analysed in clusters, a sample selection algorithm was followed. This ensured a mixture of centres, dates, times etc.

4.1.2.3.1.2 Problems identified during the quality control procedures

Each assay was registered with an external quality assurance (EQA) scheme, and assay performance was externally verified. UK Biobank used different concentrations of Quality Control (QC) samples to estimate the within-laboratory precision.

One of the issues identified during the checks was the so-called dilution issue. This happened during the initial processing of the samples when creating aliquots from the serum vacutainer. It was due to some mixing of the sample with system fluid (water); this occurred because of failed seals which could not hold a system vacuum in the handling systems.

An analysis was performed to determine the time points at which changes in mean assay levels occurred. The results over time were visually assessed and periods where “dips” in the results were observed, were excluded from the datasets (Figure 4-2).

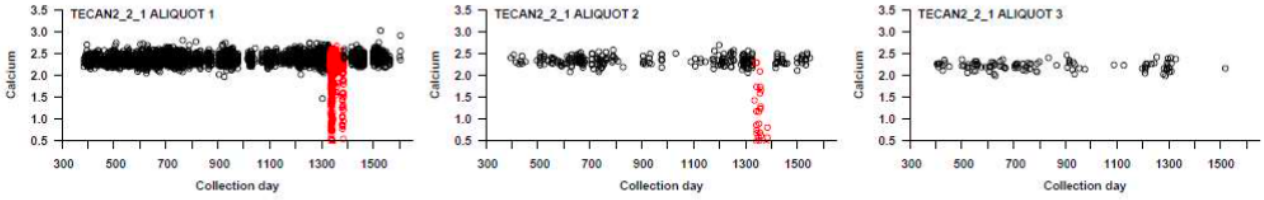


Figure 4-2: Results from calcium assays over time showing the dilution effect in aliquot 1 and 2.

These results were excluded. Image used with permission by the UK Biobank

4.1.2.4 Laboratory measurements relevant to this study

Only the measurement methods relevant to this project will be further described (calcium, albumin, vitamin D, creatinine) (Table 4-2).

4.1.2.4.1 Calcium and albumin

The measurement for these two biomarkers was done using a colourimetric assay in a Beckman Coulter AU5800 analyser. The method follows the same principle as the one performed by the Sheffield Teaching Hospitals laboratory. The only difference is that the reagent used in the case of calcium was Arsenazo III (2,2’-[1,8-Dihydroxy-3,6-disulphonaphthylene-2,7-bisazo]- bisbenzenear-sonic acid) (BeckmanCoulter, 2012; 2013)

4.1.2.4.2 Vitamin D

The LIASION 25 OH Vitamin D assay is a direct competitive chemiluminescence immunoassay (CLIA). During the first incubation, 25(OH)D is dissociated from its binding protein and binds to the specific antibody on the solid phase. The tracer (vitamin D linked to an isoluminol derivative) is added after ten minutes. After a second ten-minute incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added to initiate a flash chemiluminescent reaction. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25(OH) vitamin D present in calibrators, controls, or samples (Diasorin, 2013).

4.1.2.4.3 Creatinine

The method followed to measure creatinine was an enzymatic one. This method utilizes a multi-step approach ending with a photometric end-point reaction. The enzyme creatinine amidohydrolase is used to convert creatinine to creatine. Creatine is in turn hydrolysed by creatinine amidohydrolase to sarcosine and urea. Sarcosine from this reaction is oxidized by sarcosine oxidase to glycine and formaldehyde. This reaction also produces hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and N-ethyl-N-(3-sulfopropyl)-3-methylaniline in the presence of peroxidase to yield a coloured chromogen read at 560 nm (quinoneimine dye). The resulting change in absorbance is proportional to the creatinine concentration in the sample (BeckmanCoulter, 2015).

	ICQ level	ICQ level	Average within laboratory CV	Average SD
	Low	1.44-1.66	1.61	0.03

Calcium (mmol/L)	Medium	2.29-2.58	1.39	0.03
	High	2.96-3.30	1.29	0.04
Albumin (g/L)	Low	27.1-33.5	2.09	0.63
	Medium	41.8-49.7	2.20	1.00
	High	50-61.2	2.13	1.18
Vitamin D (nmol/L)	Low	26.0-49.9	6.14	2.21
	Medium	54.2-85.9	5.39	3.81
	High	78.1-116	5.04	4.73
Creatinine (µmol/L)	Low	55-68	2.75	1.71
	Medium	159-197	1.91	2.80
	High	470-529	1.39	6.92

Table 4-2: Internal Quality Control (ICQ) levels and within laboratory coefficient of variation (CV) given by the UK Biobank

4.1.3 Method development

4.1.3.1 Application and databases

In order for a researcher to apply for UK Biobank data, a registration has to be completed, followed by an application. This is necessary in order to assess whether the research meets legal and ethical standards, whether the study is scientifically justified and for determining the cost. If a study gets approved, the Material Transfer Agreement (MTA) gets executed and the cost is paid before the release of the data.

The University of Liverpool (Dr Fadil Hannan) in collaboration with the University of Sheffield, applied for access to the data, to complete a research project called "Biochemical evaluation of secondary osteoporosis: A UK Biobank study" (application 23448). This would compare subjects with osteoporosis, as diagnosed by the presence of low bone mineral density (BMD) values and/or fractures and identify whether they have more frequent blood test abnormalities compared to subjects without osteoporosis. These findings would establish which blood tests are of help for the clinical assessment of osteoporosis. The MTA for the study was executed on the 20th March 2018 and access to a variety of data including laboratory measurements was achieved. It was decided to use part of this data to complement the studies of the current thesis and to plan future analyses from the origins of this collaboration.

There were several datasets received after the MTA was executed and they all had to be assessed before use to identify their eligibility for use (Table 4-3). The initial databases received lacked information on all the patients and could not be used for the analysis. The ones used are shown in bold in the table (Table 4-3).

Database	Information	Date released	Number of patients
21553	General information	19/04/2018	2178

22444	General information	01/06/2018	54091
27608	General information	10/04/2019	502536
29169 **29859**	Biochemical measurements	17/04/2019 10/05/2019	All patients
37987	General information	27/09/2019	502524

Table 4-3: Table of databases received from UK Biobank and were assessed for this study.

Some databases were refreshed to include more updated data or data from a third instance (2018 onwards). The ones in bold are the ones used for the final analysis

4.1.3.2 Processing of the databases and statistical analysis

The processing of the databases received by UK Biobank and the statistical analyses have been performed using the R studio statistical software (RStudio, Inc. Boston) and Microsoft Excel (Microsoft Office).

In general, the independent student's t test was used to compare numerical values between the two assessments after checking for normality of the data using histograms. The paired t test was used when comparing the results from the same individuals on two occasions. The chi square test was used to compare categorical data.

4.1.3.2.1 Calculation of the within-subject standard deviation

As described in Chapter 2, when having multiple measurements from an individual, these measurements are generally not equal to each other and tend to vary around a "true average". Each individual then has a standard deviation of these measurements

which can easily be calculated. If there are only two measurements, the method described in Chapter 2 can be simplified. In this case, the variance is half the square of their differences (D_i: difference for two observations for subject i) and the within-subject standard deviation (S_w) equation is: $S_w^2 = \frac{1}{2k}\sum D_i^2$, k being the number of subjects. If two measurements are used, one first needs to check the assumption that the standard deviation is unrelated to the magnitude of the mean by plotting the absolute value of the difference for each subject (D_i) against the mean (Bland & Altman, 1996a).

In the case where there is a correlation, a way to overcome this issue is by log transforming the data and a plot of the log standard deviation against the log mean has to be performed. If there is no evidence of a relationship, the analysis of variance can be used to calculate the within-subject standard deviation (S_w) as described in Chapter 2. The only difference is that in this case, the calculated S_w is in a logarithmic scale, thus the antilog has to be calculated (a_{sw}). The value calculated, is not the standard deviation, but a quantity called geometric standard deviation. The coefficient of variation is the ratio of standard deviation to the mean in the original data. Thus, in logarithmic data, if we calculate the a_{sw}-1, this would be the within subject coefficient of variation (Bland & Altman, 1996b). In order to give a rough calculation of the variability of calcium, the within-standard deviation was still calculated even if this method would not overcome the evidence of a correlation. In these cases, the results have to be interpreted with caution.

4.1.3.3 Processes followed before the final analysis

The initial step was to combine all the databases together (general information database and laboratory evaluations) and assign the different codes given by the UK Biobank to the data. The code used to do this is shown in the Supplementary material (code 5.1).

4.1.3.3.1 Adjusted calcium equation

As the UK Biobank did not give an adjusted calcium equation for use, a similar approach to the one described in Chapter 2 used by the Sheffield Teaching Hospitals laboratory, was followed to calculate the equation using data from the UK Biobank. The mean of the Pathology Harmony Range was used to normalise the data (2.40 mmol/L). Unfortunately, potassium was not available at the time of the study so it was not used for the selection of patients. In terms of liver function tests, alanine transaminase (ALT) was the one available and the one used.

Therefore, out of all the patients with biochemical data, only the ones with the following characteristics were selected.

- Albumin ≤ 55 g/L and >20g\L
- Urea ≤ 15 mmol/L
- Creatinine ≤ 200 µmol/L
- ALT ≤ 41 U/L, <33 U/L for females
- Alkaline phosphatase ≤ 130 U/L

In total, 374565 patients were selected and a plot was created of total calcium (y axis) against albumin (x axis) (Figure 4-3).

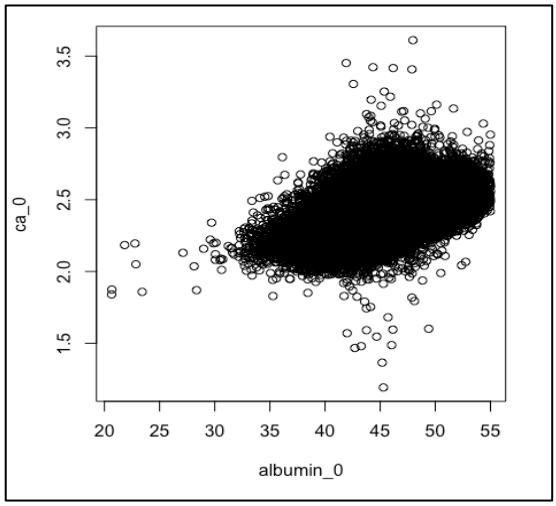


Figure 4-3: Graph of the 374565 patients selected for the calculation of the adjusted calcium equation. The y intercept was found to be 1.57822 while the slope of the graph was 0.01769

The y intercept was found to be 1.57822 while the slope of the graph was 0.01769. Thus, the equation y=(slope)x + intercept, became y = 0.0177x + 1.58. This was further processed according to the method described in Chapter 2 and the following steps were followed:

Total calcium (TCa) = 0.0177 [albumin] + 1.58

Since Adjusted calcium = total calcium - (slope * albumin) + (mean normal total calcium - intercept calcium)

Adjusted calcium (ACa) = TCa – 0.0177 [albumin] + (mean total calcium – 1.58)

If the total calcium reference interval is 2.20 – 2.60 mmol/L the mean calcium of reference interval is 2.40, then 2.40 – 1.58 = 0.82

ACa = TCa – 0.0177 [albumin] + 0.82

ACa = TCa – 0.0177 ([albumin] – 0.82/0.0177)

ACa = TCa – 0.0177 ([albumin] – 46.3)

Adjusted calcium = Total calcium + 0.0177 (46.3 – albumin)

The codes used to perform the selection of patients and the analysis can be found in the Supplementary material (code 5.2 & 5.3).

4.1.3.3.2 Glomerular Filtration Rate

As the UK Biobank did not give information on eGFR, the 2009 CKD-EPI creatinine equation was used for its calculation. The equation can then be broken down into different categories to allow a more automated calculation of eGFR (Table 4-4) (KDIGO, 2012).

Race	Gender	Creatinine (µmol/L)	Equation
Black	Female	≤62	$eGFR = 166 \times (Scr/62)^{-0.329} \times (0.993)^{age}$
Black	Female	>62	$eGFR = 166 \times (Scr/62)^{-1.209} \times (0.993)^{age}$
Black	Male	≤80	$eGFR = 163 \times (Scr/80)^{-0.411} \times (0.993)^{age}$
Black	Male	>80	$eGFR = 163 \times (Scr/80)^{-1.209} \times (0.993)^{age}$
White or other	Female	≤62	$eGFR = 144 \times (Scr/62)^{-0.329} \times (0.993)^{age}$
White or other	Female	>62	$eGFR = 144 \times (Scr/62)^{-1.209} \times (0.993)^{age}$
White or other	Male	≤80	$eGFR = 141 \times (Scr/80)^{-0.411} \times (0.993)^{age}$
White or other	Male	>80	$eGFR = 141 \times (Scr/80)^{-1.209} \times (0.993)^{age}$

Table 4-4: Different formats of the 2009 CKD-EPI equation according to race, gender and creatinine level (adapted from original (KDIGO, 2012))

These different formats were used to estimate the GFR levels to the included subjects from the UK Biobank. UK Biobank uses different codings for ethnic backgrounds and defines as “black” the following categories:

- 4: Black or Black British
- 4001: Caribbean
- 4002: African
- 4003: Any other black background

The code to complete this process can be found in the Supplementary material (code 5.3).

4.1.3.4 Definitions used

In the second part of the analysis, and in order to compare our results with the studies using data from the Metabolic Bone Centre population, I selected the participants who had normal values of eGFR (≥60 ml/min/1.73 m^2) and were vitamin D replete (≥50 nmol/L) according to the National Osteoporosis Society guidelines at baseline (Aspray et al, 2014).

The participants were first divided to different categories based on their adjusted calcium results at baseline (hypocalcaemic: AdjCa<2.20 mmol/L, normocalcaemic: AdjCa: ≥2.20 and ≤2.60 mmol/L, hypercalcaemic: AdjCa >2.60 mmol/L).

4.1.4 Results

The results are divided in two parts. First, the results of the analysis including all available participants are shown. In the second part, the results shown are from participants who had normal values of eGFR and were vitamin D replete at baseline.

The code for the first part of this analysis (including all participants) can be found in the Supplementary material (code 5.4). The code with the logarithmic transformations can also be found in the Supplementary material (code 5.5). As the codes for participants who had normal values of vitamin D and eGFR at baseline followed the same process as in codes 5.4 and 5.5, they were not included in the Supplementary material.

4.1.4.1 All participants

The study included 502524 participants, with a mean age of 57 years (SD 8.10). Out of them, 54% were female. At baseline, 429762 (86%) had adjusted calcium available (mean 2.40 mmol/L). Out of them, 1632 (0.4%) were hypocalcaemic, 6212 (1.4%) were hypercalcaemic and 421918 (98.2%) were normocalcaemic. Out of the 448368 having vitamin D values, 44.6% were vitamin D replete. Out of the 469375 with eGFR values, 97.9% had a normal level. The information on both baseline and first follow up values can be found in Table 4-5.

Groups	**Baseline**	**First follow up**	**p value**
Participants with adjusted calcium measurements, number	429762	15683	
Hypocalcaemic participants, number (%)	1632 (0.4)	38 (0.2)	0.007
Hypercalcaemic participants, number (%)	6212 (1.4)	358 (2.3)	<0.001
Normocalcaemic participants, number (%)	421918 (98.2)	15287 (97.5)	0.540

Participants with vitamin D measurements, number	448368	17039	
Participants with low vitamin D, number (%)	248235 (55.4)	9855 (57.8)	<0.001

Table 4-5: Information on the UK Biobank participants at baseline and first follow up. The p values show the statistical differences between the numbers of participants at baseline and follow up

The mean adjusted calcium at assessment 1 was 2.41 mmol/L versus 2.40mmol/L at baseline (p<0.001). The results were more marked when comparing only the measurements of individuals attending both assessments (adjusted calcium at baseline 2.39 vs 2.41mmol/L at the first follow up, p<0.001). There were similar percentages of people in the normocalcaemic category (p>0.05). However, when comparing the numbers in the hypercalcaemic category, a greater number was found at the first follow up (p<0.001). A smaller number of hypocalcaemic patients was found in the follow up (p 0.007).

Comparison of the means of other variables between the two assessments also showed statistically significant differences [total calcium (2.38 vs 2.40 mmol/L, p<0.001), eGFR (91.2 vs 86.0 ml/min/1.73m^2), 25(OH)D (49 vs 48 nmol/L, p<0.001)].

The different categories of chronic kidney disease (CKD) can be found in Table 4-6.

Chronic kidney disease stages	**Baseline Number (%)**	**First follow up Number (%)**	**p value**
G1	289988 (61.78)	8114 (45.47)	<0.001
G2	169713 (36.16)	9137 (51.20)	<0.001
G3a	7902 (1.68)	510 (2.86)	<0.001
G3b	1317 (0.28)	65 (0.36)	0.047
G4	318 (0.07)	14 (0.08)	0.695
G5	137 (0.03)	4 (0.02)	0.760

Table 4-6: Categories of chronic kidney disease in the UK Biobank population at baseline and first follow up.

G1: ≥90, G2: 60-89, G3a: eGFR 45-59, G3b: 30-44, G4: 15-29, G5: <15 ml/min/1.73 m^2 (KDIGO, 2012). In total, 469375 participants had eGFR values at baseline and 17844 at the first follow up. The p values show the statistical differences between the numbers of participants at baseline and follow up

Out of the 1632 hypocalcaemic participants at baseline, 52 had two measurements available. Only one of them remained hypocalcaemic on the second occasion (0.007% of the population with two calcium measurements, n=13419). Out of the 159 hypercalcaemic subjects with two measurements, 74 (47%) remained hypercalcaemic on the second occasion (0.55% of the population with two calcium measurements). Finally, 97% (n=12973) of the normocalcaemic participants had a persistently normal measurement of adjusted calcium.

Values	**Hypocalcaemic (n=1632)**	**Hypercalcaemic (n=6212)**	**Normocalcaemic (n=421918)**
Number of participants with two measurements	52	159	13208
Mean adjusted calcium at baseline (mmol/L)	2.15	2.68	2.40
Mean adjusted calcium at first follow up (mmol/L)	2.37	2.60	2.41
P value	<0.001	<0.001	<0.001

Table 4-7: Mean values of adjusted calcium in the three different groups studied (hypocalcaemic, hypercalcaemic and normocalcaemic).

The p values show the statistical differences between the biochemical measurements at baseline and follow up

In order to calculate the within-subject SD for each group, the absolute value of the difference for each subject against the mean was plotted. On all occasions, there was a correlation ($p<0.001$). A log transformation of the data was attempted as described in the methods. This did not change the existing correlations for the hypocalcaemic and hypocalcaemic groups ($p<0.001$), but it changed the correlation for the normocalcaemic group ($p>0.05$). The calculated coefficient of variation for the

normocalcaemic group was 3.3%. Although the assumptions were not met, the coefficient of variation for the hypocalcaemic and hypercalcaemic groups were calculated, in order to get an estimate of their magnitudes. The values were 7.3% and 4.7% respectively (Table 4-10).

A further calculation was performed for the persistent hypercalcaemic (n=74) and the persistent normocalcaemic (n=12973). The coefficients of variation were 3.4% and 3.1% respectively.

4.1.4.2 Vitamin D replete participants with normal eGFR

The next step was to select participants with normal eGFR who were also vitamin D replete at baseline. This included 178377 participants (35% of the total population), with a mean age of 57 years (SD 8.01). Out of them, 54% were female. Table 4-8 shows the information on both baseline and first follow up values.

Groups with normal vitamin D and eGFR at their baseline evaluation	**Baseline**	**First follow up**	**p value**
Participants with adjusted calcium measurements, number	178377	6406	
Hypocalcaemic participants, number (%)	512 (0.3)	12 (0.2)	0.249
Hypercalcaemic participants, number (%)	2358 (1.3)	125 (2.1)	<0.001
Normocalcaemic participants, number (%)	175507 (98.4)	6744 (97.7)	0.704

Table 4-8: Information on the UK Biobank participants who had normal vitamin D and eGFR at their baseline evaluation.

The p values show the statistical differences between the numbers of participants at baseline and follow up

Out of the 512 hypocalcaemic participants at baseline, 18 had two measurements available. None of one of them remained hypocalcaemic on the second occasion. Out

of the 2358 hypercalcaemic patients at baseline, 61 had two measurements and 24 (39%) remained hypercalcaemic on the second occasion. Finally, 98% of the normocalcaemic participants had a persistently normal measurement of adjusted calcium (Table 4-9).

The mean adjusted calcium at assessment 1 was 2.42 mmol/L versus 2.40 mmol/L at baseline ($p<0.001$). There were similar percentages of people in the hypocalcaemic and normocalcaemic category ($p>0.05$). However, when comparing the numbers in the hypercalcaemic category, a greater number was found at the first follow up ($p<0.001$).

Values	**Hypocalcaemic (n=512)**	**Hypercalcaemic (n=2358)**	**Normocalcaemic (n=175507)**
Number of participants with two measurements	18	61	5802
Mean adjusted calcium at baseline (mmol/L)	2.15	2.67	2.39
Mean adjusted calcium at first follow up (mmol/L)	2.37	2.59	2.42
P value	<0.001	<0.001	<0.001

Table 4-9: Mean values of adjusted calcium in the three different groups studied (hypocalcaemic, hypercalcaemic and normocalcaemic).

This table only contains information on participants who had normal vitamin D and eGFR at their baseline evaluation. The p values show the statistical differences between the biochemical measurements at baseline and follow up

In order to calculate the within-subject SD for each group, the absolute value of the difference for each subject against the mean was plotted. On all occasions, there was a correlation ($p<0.001$). A log transformation of the data was attempted as described in the methods. This did not change the existing correlations for the hypocalcaemic and hypocalcaemic groups ($p<0.001$), but it changed the correlation for the

normocalcaemic group (p>0.05). The calculated coefficient of variation for the normocalcaemic group was 3.2%. Although the assumptions were not met, the coefficient of variation for the hypocalcaemic and hypercalcaemic groups were calculated, in order to get an estimate of their magnitudes. The values were 6.1% and 4.2% respectively, these slightly lower from the population with all values of vitamin D and eGFR included (Table 4-10).

CV, %	Hypocalcaemic	Hypercalcaemic	Normocalcaemic
All	7.3 (N)	4.7 (N)	3.3 (Y)
VitD and eGFR normal	6.1 (N)	4.2 (N)	3.2 (Y)

Table 4-10: Table of the coefficients of variation (CV) calculated for the different groups.

The groups were defined according to the adjusted calcium measurements at baseline. Y (yes) or N (no) indicates whether assumptions for the tests were met. All the calculations were performed after log transforming the data

4.1.5 Discussion

I have provided some information on the variability of adjusted calcium in the general population, using data from the UK Biobank. Regardless of vitamin D and CKD status, hypocalcaemic patients seem to have the greatest variability, followed by the hypercalcaemic ones. The majority of normocalcaemic subjects kept a persistent normal value of adjusted calcium, although their mean measurement increased over time.

The mean adjusted calcium at baseline was 2.39 mmol/L versus 2.41 mmol/L at the first follow up (p<0.001) for the participants that attended both assessments. This

increase could be due to biological or methodological reasons. Some of the characteristics of the participants were different at the first follow up (eg they were older), and that could have had an effect on the results, as primary hyperparathyroidism is more frequent in older individuals. Moreover, participants taking part on a study might be more self-motivated and might had started taking calcium or vitamin D supplements after their recruitment. Surprisingly, in analyses performed for this study, adjusted calcium was positively correlated with age. Moreover, the percentage of patients with CKD G2 and G3a increased at the first follow up, while the percentage of G1 decreased. A negative correlation was found with adjusted calcium and eGFR, thus the decrease in the mean eGFR at the first follow up could theoretically be part of the explanation for the increase in adjusted calcium. However, this is not what would be expected, as 1,25 dihydroxyvitamin D decreases as CKD progresses, therefore one would expect lower calcium levels. According to the UK Biobank, there was no change in the assay used over this period of time and the repeat samples were performed at the same time as the baseline ones, so any changes in the levels could not be due to a drift in the assay. The fact that the participants were not fasting could have had an impact in the results. Overall, the change in calcium is probably due to biological reasons, with PHPT being the most likely reason.

There were similar percentages of people in the hypocalcaemic and normocalcaemic category. However, when comparing the numbers in the hypercalcaemic category, a greater number was found at the first follow up ($p<0.001$). This is consistent with the increase in the level of calcium described above and probably due to an increased prevalence of PHTP.

Most of the patients originally identified as hypocalcaemic or hypercalcaemic at baseline had a normal calcium at the first follow up, with patients with low calcium having the greatest variability. There are two reasons why subjects may go from high or low to normal calcium. The first is regression to the mean which is a consequence of simple variability over time, and the second is treatment of patients found to have abnormalities at baseline (eg treatment of low calcium with supplements).

The persistence of abnormal values was higher in the hypercalcaemic group, due to the fact that causes of hypercalcaemia like cancer and primary hyperparathyroidism, can be found in older subjects. The prevalence of persistent hypocalcaemia was really low (0.006%), while the respective one for hypercalcaemia was 0.47%. This percentage is lower to previous reports but most studies only assess one measurement (Dent et al, 1987).

This study identified 0.4% hypocalcaemic patients at baseline and 1.4% hypercalcaemic (total of 1.8%). This percentage is unexpectedly low, as we would expect approximately 5% of the general population to be outside this interval. This could be due to the selection of the Pathology Harmony Range as the reference interval to use, which might be too wide. I used the data from the UK Biobank measurement at baseline to establish a reference interval for adjusted calcium. This was 2.26 to 2.56 mmol/L. The percentages of hypocalcaemia and hypercalcaemia in this case would be 1.8% and 2.9% respectively, thus the total percentage of outliers would be closer to the expected.

The strengths of this study include the fact that a large number of participants from throughout the UK was studied. Some of the limitations of this study include the fact that the subjects' ages were limited between 40 to 69 years and probably the majority

of them were in relatively good health and not confined to home or in care homes. Parathyroid hormone was not available to enable full assessment of the calcium metabolism. Only a maximum of two measurements were available, so the calculations are not so robust. On top of that, not all participants had two laboratory assessments available and there were differences in the characteristics of the participants as mentioned above which could have affected the results. Some of the samples were affected by dilution and although this was assessed in most cases, it might have had a small impact on the overall results. Figure 4-3 shows that there were some extreme values of calcium that would be incompatible with life and thus probably there were issues with the methods not completely resolved. As UK Biobank did not give an adjusted calcium equation, one had to be calculated from the given data as mentioned in a previous study (Barth et al, 1996). Potassium measurements were not available by the UK Biobank, therefore patients with low potassium could not be excluded to precisely mirror the protocol.

The implications of this analysis may become more relevant when the results of a related study have been released. The calculations and results of this project will be used in an analysis trying to identify genetic loci that regulate circulating mineral parameters such as calcium, phosphate and alkaline phosphatase. Persistent abnormalities might be more closely linked to gene polymorphisms.

Overall, this study provides a further understanding of the variability of calcium in the general population. When laboratory measurements are repeated, people with abnormal values of adjusted calcium are likely to have normal values, with the hypocalcaemic having the greatest variability.

Section 2: Comparing the UK Biobank population to the Metabolic Bone centre population

4.2.1 Introduction

Up to now, the results of the above studies at the Metabolic Bone Centre (MBC) population, have shown that persistent normocalcaemia in normocalcaemic hyperparathyroidism and hypoparathyroidism is rare and these patients often experience intermittent hypercalcaemia (NPHPT) and hypocalcaemia (NHYPO). I calculated the within-subject CV for adjusted calcium and this variability likely explains the changing of category from normal to abnormal adjusted calcium.

I wanted to find out whether the changing of category was the same in the general population as it was in the Metabolic Bone Centre and so I studied the UK Biobank.

In the UK Biobank study, it was observed that when laboratory measurements were repeated, people with abnormal values of adjusted calcium were likely to have normal values, with the hypocalcaemic having the greatest variability. However, the comparison between the MBC and the UK Biobank could not be done directly, as participants in the UK Biobank only had two measurements.

In order to check whether the same findings would be found in the MBC population, and be able to compare the two populations, extra data was requested on these patients.

4.2.2 Methods

In order to do this analysis, data of calcium and albumin after the baseline assessment were requested for all the MBC participants included in the study (n=6280). The timing

had to be after the index date and there had to be a gap of at least two years, to resemble the UK biobank gap between the two measurements. The adjusted calcium equation was calculated using the formula given in the methods section in Chapter 2.

4.2.3 Results

4.2.3.1 All participants

The MBC population had a mean age of 66 years, while the UKBB population had a mean age of 57 years (p<0.001). In the MBC population, 72% were female compared to 54% in the UKBB (p<0.001).

The percentages of hypocalcaemia, hypercalcaemia and normocalcaemia in the two populations are seen in Table 4-11. In both groups, the percentage of hypercalcaemic patients at follow up, was statistically higher than baseline. The mean adjusted calcium at baseline was 2.40 mmol/L, versus 2.41mmol/L at the first follow up (p<0.001) for the UK Biobank. The respective values for the MBC population were 2.37 and 2.42 mmol/L (p<0.001).

Groups (MBC population)	**Baseline**	**First follow up**	**p value**
Participants with adjusted calcium measurements, number	6280	2765	NA
Hypocalcaemic participants, number (%)	179 (2.9)	73 (2.6)	0.634
Hypercalcaemic participants, number (%)	245 (3.9)	227 (8.2)	<0.001
Normocalcaemic participants, number (%)	5856 (93.2)	2465 (89.2)	0.180

Groups (UKBB)	**Baseline**	**First follow up**	**p value**
Participants with adjusted calcium measurements, number	429762	15683	NA

Hypocalcaemic participants, number (%)	1632 (0.4)	38 (0.2)	0.007
Hypercalcaemic participants, number (%)	6212 (1.4)	358 (2.3)	<0.001
Normocalcaemic participants, number (%)	421918 (98.2)	15287 (97.5)	0.540

Table 4-11: Information at baseline and first follow up for the MBC population (top) and the UK Biobank population (below).

The p values show the statistical differences between the numbers of participants at baseline and follow up. In both groups, The percentage of hypercalcaemic patients at follow up, is statistically higher than baseline. That is probably due to the fact that subjects with high calcium are more likely to have their calcium repeated and the mean calcium at follow up is larger than at baseline

The persistence of the laboratory investigations throughout the follow up was checked. Using the UK Biobank data, 2% of the hypocalcaemic patients remained in the same category, 47% of the hypercalcaemic and 98% of the normocalcaemic. The respective percentages for the MBC population were 15%, 58% and 92% (Table 4-13). The consistency of hypocalcaemia was statistically greater in the MBC population (p 0.042), while the other categories did not have significant differences.

Values (referral)	**Hypocalcaemic at baseline (n=179)**	**Hypercalcaemic at baseline (n=245)**	**Normocalcaemic at baseline (n=5856)**
Number of participants with two measurements	91	154	2520
Mean adjusted calcium at baseline (mmol/L)	2.14	2.74	2.37
Mean adjusted calcium at first follow up (mmol/L)	2.30	2.63	2.41
P value	<0.001	<0.001	<0.001

Values (UKBB)	**Hypocalcaemic at baseline (n=1632)**	**Hypercalcaemic at baseline (n=6212)**	**Normocalcaemic at baseline (n=421918)**
Number of participants with two measurements	52	159	13208

Mean adjusted calcium at baseline (mmol/L)	2.15	2.68	2.40
Mean adjusted calcium at first follow up (mmol/L)	2.37	2.60	2.41
P value	<0.001	<0.001	<0.001

Table 4-12: Mean values of adjusted calcium in the three different groups studied (hypocalcaemic, hypercalcaemic and normocalcaemic).

The numbers included are for subjects that had two measurements available. The top table shows data from the MBC population, while the bottom one shows data from the UK Biobank. The p values show the statistical differences between the biochemical measurements at baseline and follow up

Values (MBC population)	**Hypocalcaemic at baseline (n=179)**	**Hypercalcaemic at baseline (n=245)**	**Normocalcaemic at baseline (n=5856)**
Number of participants having two measurements	91	154	2520
Number of subjects that remained in the same category at follow up (%)	14 (15)	89 (58)	2328 (92)
Values (UKBB)	**Hypocalcaemic at baseline (n=1632)**	**Hypercalcaemic at baseline (n=6212)**	**Normocalcaemic at baseline (n=421918)**
Number of participants having two measurements	52	159	13208
Number of subjects that remained in the same category at follow up (%)	1 (2)	74 (47)	12942 (98)
P value	**0.042**	**0.307**	**0.062**

Table 4-13: Table showing the number of subjects in each category (hypocalcaemic, hypercalcaemic and normocalcaemic) at baseline and follow up.

The number of subjects remaining on the same category at follow up are then compared.

In order to calculate the within-subject SD for each group, the absolute value of the difference for each subject against the mean was plotted. On all occasions, there was a correlation ($p<0.001$). A log transformation of the data was attempted as described in the methods. This did not change the existing correlations for the hypocalcaemic

and hypocalcaemic groups (p<0.001), but it changed the correlation for the normocalcaemic group (p>0.05). The calculated coefficient of variation for the normocalcaemic group was 3.5% (UKBB 3.2%). Although the assumptions were not met, I also calculated the coefficient of variation for the hypocalcaemic and hypercalcaemic groups to get an estimate of their magnitudes. The values were 7.5% and 6.7% respectively, The values from the UK Biobank analysis were 7.3 and 4.7% respectively (Table 4-17).

4.2.3.2 Vitamin D replete participants with normal eGFR

To start with, the percentages of hypocalcaemia, hypercalcaemia and normocalcaemia in the two populations are seen in Table 4-11. In both groups, the percentage of hypercalcaemic patients at follow up, was statistically higher than baseline.

Groups (MBC population)	**Baseline**	**First follow up**	**p value**
Participants with adjusted calcium measurements, number	2451	1045	NA
Hypocalcaemic participants, number (%)	55 (2.24)	31 (2.97)	0.219
Hypercalcaemic participants, number (%)	57 (2.33)	63 (6.03)	<0.001
Normocalcaemic participants, number (%)	2339 (95.43)	951 (91.00)	0.373

Groups (UKBB)	**Baseline**	**First follow up**	**p value**
Participants with adjusted calcium measurements, number	178377	6406	
Hypocalcaemic participants, number (%)	512 (0.3)	12 (0.2)	0.249
Hypercalcaemic participants, number (%)	2358 (1.3)	125 (2.1)	<0.001

Normocalcaemic participants, number (%)	175507 (98.4)	6744 (97.7)	0.704

Table 4-14: Information at baseline and first follow up for the MBC population (top) and the UK Biobank population (below).

The p values show the statistical differences between the numbers of participants at baseline and follow up. In both groups, The percentage of hypercalcaemic patients at follow up, is statistically higher than baseline. That is probably due to the fact that subjects with high calcium are more likely to have their calcium repeated and the mean calcium at follow up is larger than at baseline

The persistence of the laboratory investigations throughout the follow up was checked. Using the UK Biobank data, 0% of the hypocalcaemic remained in the same category, 39% of the hypercalcaemic and 99% of the normocalcaemic. The respective percentages for the MBC population were 18%, 57% and 93% (Table 4-16).

Values (referral)	**Hypocalcaemic at baseline (n=55)**	**Hypercalcaemic at baseline (n=57)**	**Normocalcaemic at baseline (n=2339)**
Number of participants with two measurements	27	35	983
Mean adjusted calcium at baseline (mmol/L)	2.13	2.74	2.38
Mean adjusted calcium at first follow up (mmol/L)	2.17	2.66	2.40
P value	0.006	0.009	<0.001

Values (UKBB)	**Hypocalcaemic (n=512)**	**Hypercalcaemic (n=2358)**	**Normocalcaemic (n=175507)**
Number of participants with two measurements	18	61	5802
Mean adjusted calcium at baseline (mmol/L)	2.15	2.67	2.39
Mean adjusted calcium at first follow up (mmol/L)	2.37	2.59	2.42
P value	<0.001	<0.001	<0.001

Table 4-15: Mean values of adjusted calcium in the three different groups studied (hypocalcaemic, hypercalcaemic and normocalcaemic).

The numbers included are for subjects that had two measurements available. The top table shows data from the MBC population, while the bottom one shows data from the UK Biobank. The p values show the statistical differences between the biochemical measurements at baseline and follow up

Values (MBC population)	**Hypocalcaemic at baseline (n=55)**	**Hypercalcaemic at baseline (n=57)**	**Normocalcaemic at baseline (n=2339)**
Number of participants having two measurements	27	35	983
Number of subjects that remained in the same category at follow up (%)	5 (18.5)	20 (57.1)	914 (93.0)
Values (UKBB)	**Hypocalcaemic at baseline (n=512)**	**Hypercalcaemic at baseline (n=2358)**	**Normocalcaemic at baseline (n=175507)**
Number of participants having two measurements	18	61	5802
Number of subjects that remained in the same category at follow up (%)	0 (0.0)	24 (39.3)	5744 (99.0)
P value	0.202	0.312	0.206

Table 4-16: Table showing the number of subjects in each category (hypocalcaemic, hypercalcaemic and normocalcaemic) at baseline and follow up.

The number of subjects remaining on the same category at follow up are then compared.

In order to calculate the within-subject SD for each group for the MBC population, the absolute value of the difference for each subject against the mean was plotted. In all the occasions, there was a correlation ($p<0.001$). I then tried doing the same by log transforming the data as described in the methods. This changed the existing correlations for all the groups ($p>0.05$). The calculated coefficient of variation for the

hypocalcaemic group was 5.5% (6.1% for UKBB), for the hypercalcaemic 5.5% (4.2% for UKBB) and 3.3% for the normocalcaemic (UKBB 3.2%) (Table 4-17).

CV, %	Hypocalcaemic	Hypercalcaemic	Normocalcaemic
UKBB: All	7.3 (N)	4.7 (N)	3.3 (Y)
VitD and eGFR normal	6.1 (N)	4.2 (N)	3.2 (Y)
Referral: All	7.5 (N)	6.7 (N)	3.5 (Y)
VitD and eGFR normal	5.5 (Y)	5.5 (Y)	3.3 (Y)

Table 4-17: Table of the coefficients of variation (CV) calculated for the different groups.

The groups were defined according to the adjusted calcium measurements at baseline. Y (yes) or N (no) indicates whether assumptions for the tests were met. All the calculations were performed after log transforming the data

4.2.4 Discussion

Overall, I observed similar patterns in the two populations (the hypocalcaemic patients were more variable followed by the hypercalcaemic ones), although the characteristics of the two groups were significantly different (older and more likely to be female in the MBC population).

In both groups, the percentage of hypercalcaemic patients at follow up was statistically higher at follow-up than baseline. For the MBC population, this is probably due to the fact that subjects with high calcium are more likely to have their calcium repeated. Moreover, in both populations, the mean calcium at follow up was greater than at baseline, with MBC having the greatest increase which could explain the greater percentage of hypercalcaemia at the first follow up.

The consistency of hypocalcaemia was statistically greater in the MBC population (p 0.042) when analysing the whole cohort, while the other categories did not have significant differences. That is probably due to the fact that truly diseased patients are more likely to be found in a MBC population. On the contrary, the general population can have incidental extreme values which then return to normal. This could be due to simple variability over time, or even treatment of patients found to have abnormalities at the first visit.

Although not all the assumptions were met and the results have to be taken with caution, the variability of calcium in the in both populations was greater in the hypocalcaemic participants, followed by the hypercalcaemic. These percentages followed the same pattern when only vitamin D replete participants with normal eGFR were included, although the results were decreased, which means that part of the variability could be due to vitamin D and eGFR variations as expected. Not surprisingly, the coefficient of variation that was not similar between the two groups, was the one of the hypercalcaemic participants; the MBC hypercalcaemic participants had a higher CV (6.7% vs 4.7%). This result probably shows that there is an increased likelihood of extreme values in a MBC population. These differences cannot be explained by different preparation of the patients, as in both occasions the individuals were not fasting and the samples were taken at any point of the day.

The clinical implications of this study will be discussed further in the final chapter.

Section 3: Reference range for adjusted calcium

4.3.1 Introduction

As seen above, the UK Biobank study identified 0.4% hypocalcaemic patients at baseline and 1.4% hypercalcaemic (total of 1.8%). This percentage is unexpectedly low, as we would expect approximately 5% of the general population to be outside this interval. This could be due to the selection of the Pathology Harmony Range as the reference interval to use, which might be too wide.

In order to check how the results would differ by using a different reference interval, the data from the UK Biobank were used to establish a population reference range for adjusted calcium.

4.3.2 Methods

In order to establish the UK Biobank reference range, all participants with a measurement of adjusted calcium at baseline were included. Exclusion criteria were low eGFR (<60 ml/min/m^2) and/or low vitamin D (<50 nmol/L) (Group 1).

The analysis was done using the results from the adjusted calcium calculated with the equation derived from the UK Biobank: Adjusted calcium = Total calcium + 0.0177 (46.3 – albumin). A second analysis was done using the standard equation for adjusted calcium: Adjusted calcium =Total calcium + 0.02 (40-albumin) (Crowley & Gittoes, 2016).

The analysis was performed using the reference range function of MedCalc Software (Belgium). The data were normally distributed, so the normal approach was used for

the calculation of the intervals. Reed's criterion was used to define outliers. This evaluates the difference between the extreme value and the next highest (or lowest). The extreme value is then rejected if this difference is more than one-third of the absolute range of values (highest minus lowest) (Fraser, 2013). Any further calculations were performed using the R studio statistical software (RStudio, Inc. Boston).

4.3.2.1 Validation analysis

According to literature, data mining is an indirect method for establishing reference intervals. This is done by using large databases obtained from the laboratory. The caveat of doing so, is that no clinical information is usually available on these patients and so, especially when dealing with hospital data, the population would include two subpopulations: the "healthy" and the "non-healthy". Each subpopulation would then have its own distribution of data and the distribution of the "non-healthy" can be excluded before calculating the reference intervals. There are several mathematical approaches to calculate these intervals (Ferré-Masferrer et al, 1999; Labrtorian, 2017).

Although the UK Biobank population is part of the general population and not part of the hospital population, so presumably they are healthier, I decided to use one of these analyses in order to check the reference interval obtained in analysis 1.

In this analysis, I included all the participants with a measurement of adjusted calcium at baseline without any exclusions (Group 2). I only calculated the reference interval for the adjusted calcium calculated with the equation derived from the UK Biobank: Adjusted calcium = Total calcium + 0.0177 (46.3 – albumin).

The analysis was performed using the R studio statistical software (RStudio, Inc. Boston) according to a previously used code (Labrtorian, 2017).

4.3.3 Results

In total, 178377 participants with available calcium measurements, normal eGFR and who were also vitamin D replete at baseline were included [mean age of 57 years (SD 8.01)]. Out of them, 54% were female.

The calculated reference interval was 2.25 to 2.56 mmol/L (Table 4-18). The percentage of subjects with hypocalcaemia and hypercalcaemia in this case would be 1.4% and 2.8% respectively, thus the total percentage of outliers would be closer to the expected. Using the standard equation for adjusted calcium, the respective percentages would be 0.05% and 26.75%.

	Adjusted calcium using calculated equation (mmol/L)	Adjusted calcium using standard equation (mmol/L)	Total calcium (mmol/L)
Lower limit	2.247	2.123	2.205
90% confidence intervals	2.246 to 2.247	2.122 to 2.123	2.205 to 2.206
Higher limit	2.558	2.435	2.564
90% confidence intervals	2.558 to 2.559	2.435 to 2.436	2.563 to 2.564
Mean (SD)	2.40 (0.079)	2.27 (0.079)	2.38 (0.094)

Table 4-18: Table with calculated reference intervals for adjusted calcium and total calcium.

The second column shows the results of the adjusted calcium calculated with the equation derived from the UK Biobank, while the third column provides data from the adjusted calcium calculated with the standard equation: Adjusted calcium =Total calcium + 0.02 (40-albumin) (Crowley & Gittoes, 2016)

4.3.3.1 Validation analysis

At baseline, 429762 participants had adjusted calcium available (mean 2.40 mmol/L). The results of the different analyses are shown below (Table 4-19). As the results of this analysis are based on visual assumptions to which population is the healthy and the unhealthy, the results were only used for validation purposes (Figure 4-4).

Adjusted calcium (mmol/L)	Healthy		Non-healthy	
	Lower limit	Upper limit	Lower limit	Upper limit
Gaussian Mixture Model, n=429762	2.25	2.53	2.19	2.75
Analysis 1, n=178377	2.25	2.56	NA	NA

Table 4-19: Results from the reference interval analysis using one of the data mining mathematical approaches for defining the healthy population.

This is compared with the results from analysis 1. NA: not available

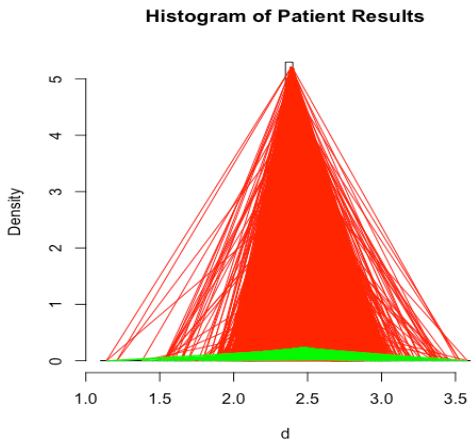


Figure 4-4: Results from the histogram showing the "healthy" (red) and "non-healthy" (green) subgroup distributions

4.3.4 Discussion

After studying the large dataset of the UK Biobank, my conclusion is that probably the Pathology Harmony Range for adjusted calcium is not the ideal to use, although it has the benefit of being comparable between laboratories. The calculated reference interval for this population was 2.25 to 2.56 mmol/L. Interestingly, the confidence intervals of the lower and upper limit did not include the current limits of 2.20 and 2.60 mmol/L. This interval would give a percentage of outliers that is closer to the expected one. Interestingly, the results from the mathematical approach that divides participants into "healthy" and "non-healthy" subgroups, were similar (reference interval 2.25-2.53 mmol/L).

This observation has clinical consequences. Moving the reference range to either direction would affect the number of individuals that are labelled as having a disorder of calcium metabolism. In the case of normocalcaemic hyperparathyroidism studied in Chapter 3, it was interesting to see that after carefully evaluating these patients at baseline, their adjusted calcium levels were between 2.49 to 2.60 mmol/L, thus in the upper normal range for calcium. A change of the upper limit of the reference range would include at least some of these patients in the primary hyperparathyroidism group. In more detail, out of the 11 NPHPT patients identified, six (55%) would had been classified as PHPT, as they had a calcium higher than 2.56 mmol/L. Out of these, only one would be persistently normocalcaemic throughout the follow up (S0911). This patient did not have persistently high PTH as seen in Chapter 3.

A topic of debate in the latest literature, has been whether clinicians should use the albumin-adjusted calcium or not. This was addressed by a recent study performed in Norway where the researchers evaluated the laboratory tests of 6549 patients (two years or older, both inpatient and outpatient) from 2006 to 2015. These patients had free (ionised) calcium measured with an ion-selective electrode in a blood analyser and standardised at a pH of 7.40. They then divided their population into two subgroups: patients with eGFR<60 ml/min/1.73m^2 and patients with eGFR≥60 ml/min/1.73m^2. They divided the second subgroup further to the ones having albumin below or above 30g/L, as their local scatterplots of total calcium against showed linearity above and below this level. They then created different equations for the three subgroups based on the following equation: Adjusted calcium = calcium + coefficient × (40−albumin). The relevant coefficients were estimated using multiple linear regression; total calcium was the dependent variable, while free calcium, albumin, phosphate, eGFR, gender, age and hospitalisation (or not) as the explanatory variables. In order to resemble the approach used in other labs and this study, they also performed a simple linear regression analysis with total calcium (dependent variable) and albumin (explanatory variable). The results of the coefficients were different than the ones with simple linear regression. This provided the unadjusted albumin coefficients. Then, they assessed the diagnostic accuracy of the equations using ROC curves and ionised calcium as their gold standard. They also assessed previously published equations. Overall, they found that the unadjusted calcium was not inferior to any of the adjusted formulas. In more detail, it performed better in patients with decreased eGFR, but when diagnosing hypercalcaemia some of the adjusted calcium equations performed better. In their limitations they mentioned that they could only use the pH-adjusted free calcium and not the actual one. They also

did not have any clinical information on these patients. Their recommendation is to measure ionised calcium (Lian & Åsberg, 2018). However, as pointed out in the introduction of the thesis, ionised calcium is not reliable and its measurement has several problems. Measuring the total calcium and albumin and then adjusting is the recommended approach in the UK and I followed this recommendation (NICE, 2019).

This chapter raises another issue. A lot of the studies use previously published equations for adjusted calcium as the one mentioned above, in order to establish their prevalence of NPHPT. I have shown, that when evaluating patients for disorders of calcium metabolism calcium, a derived regression equation based on the local population should be used as the published one might not perform as well. This was suggested previously (Crowley & Gittoes, 2016).

Overall, probably the Pathology Harmony Range for adjusted calcium is not the ideal to use, although it has the benefit of being comparable between laboratories. The calculated reference interval for the UK Biobank population was 2.25 to 2.56 mmol/L.

Section 4: Further analyses

After the completion of the previous analyses, I realised that using the mean of the Pathology Harmony range when calculating the adjusted calcium equation was probably wrong. The mean of the total calcium of the population would had been a better option.

Therefore, using the mean of total calcium of the population described in Table 4-18 (2.38 mmol/L), I followed the steps described below to get the updated adjusted calcium equation:

Total calcium (TCa) = 0.0177 [albumin] + 1.58

Since Adjusted calcium = total calcium - (slope * albumin) + (mean normal total calcium - intercept calcium)

Adjusted calcium (ACa) = TCa – 0.0177 [albumin] + (mean total calcium – 1.58)

If the total calcium reference interval is 2.21 – 2.56 mmol/L (Table 4-18), the mean calcium of reference interval is 2.38, then 2.38 – 1.58 = 0.80

ACa = TCa – 0.0177 [albumin] + 0.82

ACa = TCa – 0.0177 ([albumin] – 0.80/0.0177)

ACa = TCa – 0.0177 ([albumin] – 45.2)

Adjusted calcium = Total calcium + 0.0177 (45.2 – albumin)

Using this equation, the results of the above mentioned table are to be adjusted to the following.

	Adjusted calcium using updated calculated equation (mmol/L)	Adjusted calcium using standard equation (mmol/L)	Total calcium (mmol/L)
Lower limit	2.227	2.123	2.205
90% confidence intervals	2.227 to 2.228	2.122 to 2.123	2.205 to 2.206
Higher limit	2.539	2.435	2.564
90% confidence intervals	2.538 to 2.540	2.435 to 2.436	2.563 to 2.564
Mean (SD)	2.38 (0.079)	2.27 (0.079)	2.38 (0.094)

Table 4-20b: Table with calculated reference intervals for adjusted calcium and total calcium.

The second column shows the results of the adjusted calcium calculated with the equation derived from the UK Biobank, while the third column provides data from the adjusted calcium calculated with the standard equation: Adjusted calcium =Total calcium + 0.02 (40-albumin) (Crowley & Gittoes, 2016)

Using this updated reference interval of adjusted calcium (2.23 to 2.54 mmol/L), 0.96% would be classified as hypocalcaemic and 4.15 as hypercalcaemic. The results of the Gaussian mixture model analysis in this case would be 2.24 to 2.51 mmol/L.

For total calcium (calculated reference range 2.21 to 2.56 mmol/L), the results of the Gaussian mixture model analysis would be 2.21 to 2.56 mmol/L

Chapter 5: Discussion and future plans

Section 1: Metabolic Bone Centre studies

Section 2: UK Biobank studies

Section 3: Future plans

This study has focused on studying two newly described disorders of calcium metabolism, normocalcaemic hyperparathyroidism (NPHPT) and normocalcaemic hypoparathyroidism (NHYPO). Using data from a tertiary metabolic bone centre, it addressed the issues of the prevalence, natural history and variability of adjusted calcium.

A summary of the findings from each chapter is provided below along with a short discussion around the main challenges, strengths and limitations of the study and its clinical implications.

Section 1: Metabolic Bone Centre studies

5.1.1 Normocalcaemic hyperparathyroidism

5.1.1.1 Summary of findings

According to the proceedings of the Fourth International Workshop on Asymptomatic Primary Hyperparathyroidism, NPHPT remains incompletely described, especially regarding its epidemiology, natural history and management (Silverberg et al, 2014). The prevalence of NPHPT described in the literature varies significantly and as described in Chapter 3, it is reported to be between 0.1 to 8.9% (Pawlowska & Cusano, 2015). This is due to the different definitions and methodologies used in the described studies. Causes of secondary hyperparathyroidism were not always excluded and some studies did not address the issue of persistence of laboratory investigations as defined by the international guidelines on asymptomatic primary hyperparathyroidism (PHPT) (Eastell et al, 2014). Instead, they used data from baseline visits and thus might have overestimated the prevalence.

Trying to address the issues from the previous studies, I have studied the prevalence in a referral population having access to follow-up measurements for these patients over a period of up to five years. I used data from 6280 patients attending the Metabolic Bone Centre in Sheffield Teaching Hospitals National Health Service Foundation Trust (STH NHS FT) in the United Kingdom. Based on the baseline characteristics, I have excluded causes of secondary hyperparathyroidism. These were medications known to affect parathyroid hormone (PTH) levels (diuretics, lithium, denosumab, bisphosphonates, anticonvulsants, phosphorus), low vitamin D (<50nmol/L), chronic kidney disease (eGFR <60 ml/min/1.73m^2), renal calcium loss (hypercalciuria) and diseases of the gastrointestinal tract known to affect calcium absorption (coeliac disease, inflammatory bowel disease, bariatric surgery) (Eastell et al, 2009; Eastell et al, 2014; Yacobi-Bach et al, 2015). I then checked for persistence of adjusted calcium on at least two occasions and any patients not fulfilling this criterion were excluded. In total, thirteen patients were identified as having NPHPT. The prevalence was 0.18%. The mean age of these patients was 69 years, and 91% were female.

The results for the natural history remain controversial with some studies reporting no conversion to hypercalcemia (Ayturk et al, 2006; Garcia-Martin et al, 2012; Tordjman et al, 2004), while others report a small percentage of patients becoming hypercalcemic (Cusano et al, 2013a; Diri et al, 2014; Silverberg & Bilezikian, 2003; Siprova et al, 2016). The problem with the studies on natural history is that, although they might check persistence at baseline on two to three occasions, they then check the laboratory measurements on only one occasion after a certain period; if at that point the patient has high calcium, they conclude that the patient has progressed to hypercalcemia. I believe that this approach is not the correct one, as the hypercalcaemia may be intermittent.

Therefore, I studied the NPHPT patients further by looking at their follow up measurements and I identified two patterns, persistent normocalcaemia and intermittent hypercalcaemia. Intermittent hypercalcaemia was the common pattern, occurring in 7 out of 11 patients. Persistent normocalcemia was uncommon and only occurred in four patients (0.06% of the whole population). However, only two of these patients had consistently high PTH, but they were not consistently vitamin D replete or did not have consistently normal eGFR. If the international guidelines were strictly applied, the prevalence of NPHPT in this population would be zero.

At the time of this study, this finding of intermittent hypercalcaemia in these patients, had only been reported in one previous study from the Czech Republic, who followed up 187 patients with NPHPT for one to seven years. Out of them, 36% had intermittent hypercalcemia (Siprova et al, 2016). After the completion of our study, a further study evaluating NPHPT patients going through parathyroidectomy, reported that 40.8% of these patients had episodes of hypercalcaemia >1 year prior to surgery (Sho et al, 2019).

My study suggests that NPHPT is probably a variant of PHPT, as several PHPT patients in this study also had intermittent hypercalcaemia and the variability of adjusted calcium found, was similar in these two groups and slightly higher than the control group. This theory contradicts the one described before, that NPHPT is the first phase of a biphasic disease course, which can be followed by hypercalcemic hyperparathyroidism (Silverberg & Bilezikian, 2003).

In conclusion, this study describes the largest population ever studied to identify NPHPT and provides data during a long follow up period (five years). The prevalence reported is small and persistent normocalcemia in patients with NPHPT is rare. This

study is the first that evaluates the natural history of patients with NPHPT in so much detail and provides data from a long follow up period. Subsequent hypercalcemia in these patients should be treated with caution, and not be automatically addressed as progression to hypercalcaemia. I suggest that the definition should be revisited and normocalcemic hyperparathyroidism should be defined as having the average calcium within normal limits and not persistently normal.

5.1.1.2 Clinical relevance

Patients with elevated PTH and normal calcium are frequently assessed in referral centres and the question remains to whether they should be followed up or treated with surgery according to the PHPT guidelines. From my clinical experience, these patients are discussed in meetings, labelled as normocalcaemic hyperparathyroidism at first and then maybe discharged to their general practitioners for follow up with the instruction to be referred back in case of elevated calcium. However, elevated calcium at least on some occasions, could be part of their natural history. This variability complicates their monitoring. I have provided estimates of variability for these groups which can be used in everyday practice for monitoring. I have provided standard estimates of the within-subject SD but also more conservative ones using the 90th centile of the variance. The least significant change given, could be used in two ways in these patients. First, it gives a range to which values are expected. For example, for a patient with NPHPT having an adjusted Ca of 2.55 mmol/L, the LSC is 0.25, so the next measurement would lie between 2.30 and 2.80 mmol/L, hence could be high. This can provide some reassurance for the patient and the physician. Moreover, it can be a marker of whether an individual has an actual significant change of their calcium.

Some patients with NPHPT are being operated and using the given LSC estimates, the physician can make sure that there was a statistically significant drop in calcium as a result of the parathyroidectomy.

Identifying patients with NPHPT and excluding causes of secondary hyperparathyroidism based on the international criteria was sometimes challenging for this research project and I anticipate similar challenges in the everyday clinical practice. Patients who had vitamin D deficiency at some point during these five years, were difficult to assess, as the subsequent finding of high PTH after correcting the deficiency could had been a true finding of NPHPT or a consequence of chronic vitamin D deficiency. It has been reported in literature, that even several months after vitamin D replacement, PTH remains elevated in a percentage of people. Shibli-Rahhal et al performed a retrospective analysis and reported that after an average of ten months of treatment with different protocols, only 44% of the patients achieving 25(OH)D≥ 30ng/ml had normalised their PTH. They conclude their study by saying that the use of PTH to monitor the response to therapy is limited (Shibli-Rahhal & Paturi, 2014). Another group randomised 64 postmenopausal women to either 300000 IU colecalciferol at baseline and at three months or 1000 IU per day and had measurements at three and at six months. Some of the subjects that achieved a 25(OH)D ≥30 ng/ml, still had a PTH above the reference interval (13.3% in the 1000 IU and 17% in the 300000 IU group) (Giusti et al, 2010). Presumably these patients have autonomous parathyroid function as a result of parathyroid hyperplasia from longstanding vitamin D deficiency. This could had been an issue in studies for NPHPT in which patients with vitamin D deficiency have been treated first and then re-assessed after a few months (Siprova et al, 2016). In these studies, the prevalence

might had been an overestimation. The same challenges could be faced in the clinical setting.

A further challenge which is often an issue of debating is the correct level of vitamin D deficiency. I have used a cut-off of 50 nmol/L as advised by the Royal Osteoporosis Society guidelines and the Fourth International Workshop on Asymptomatic Primary Hyperparathyroidism (Aspray et al, 2014; Eastell et al, 2014). Many researchers recommend using a higher cut-off of 75 nmol/L. I found no patients with NPHPT using this cut-off. If I had used a higher one, it would not had made any difference in the results.

Furthermore, when estimating the persistence of a disease using databases, certain filters need to be applied in the software used. Therefore, these cut-offs in the data like vitamin D <50 nmol/L are strict and do not allow any flexibility. Therefore, patients with levels really close to these cut-offs (eg vitamin D 49 nmol/L) would still be excluded even though their PTH might not be elevated due to the vitamin D deficiency.

As the study of the prevalence of NPHPT has many challenges, that is probably one of the reasons that the reported studies vary significantly. I used a statistical approach to identify these patients and graphed the results of adjusted calcium against PTH. I observed that a lot of patients with high PTH values according to the reference intervals, were found to be “normal” by using a method for defining outliers. The subjects that were normal but had higher PTH than the reference interval, were found to be significantly older than the ones that were normal and also had normal PTH according to the current reference interval. These results align well with the fact that PTH is known to increase with age. It has been previously suggested that age-related PTH reference intervals should be established (Eastell et al, 2014). My study supports

this statement. This adjustment for the range becomes more relevant now that NPHPT is further described in literature. By using the current reference interval in everyday practice we are probably overdiagnosing NPHPT and maybe subjecting older individuals to unnecessary testing. A change of the upper limit of the reference interval in PTH could alter the number of patients diagnosed with this disorder.

A further observation after studying the large dataset of the UK Biobank, is that probably the Pathology Harmony Range is also not the ideal one to use, although it has the benefit of being widely used. It was interesting to see that after carefully evaluating the NPHPT patients at baseline, their adjusted calcium levels were between 2.49 to 2.60 mmol/L, thus in the upper normal range for calcium. A change of the upper limit of the reference range would include at least some of these patients in the PHPT group.

The question resulting from this study is whether NPHPT exists and, if it does, whether the current definition used is the most appropriate. This, obviously, is not only an issue in research studies such as the current one. This mainly affects decisions in everyday clinical practice when patients are assessed, and a diagnosis and a management plan have to be decided. Some patients might be subjected to unnecessary testing for an abnormality that is part of normal ageing. Moreover, in younger patients, the complexity of making the diagnosis might result in delayed surgery and further complications. As mentioned above, the variability described in calcium in these patients complicates their monitoring. According to the international guidelines, calcium and PTH should be tested every year, while a BMD check should be performed every one to two years. If there is a progression to hypercalcaemia, patients should be treated according to the guidelines on asymptomatic primary hyperparathyroidism (Bilezikian et al, 2014). However, a majority of these patients

have intermittent hypercalcaemia; the subsequent results might be confusing for both the care provider and the patient on whether surgery would be recommended.

I believe that there are three ways forward to the issue of diagnosing NPHPT. If we adjust the reference interval for PTH for age, and use the current definition, NPHPT may not exist, as shown in this study, at least in older individuals. Another approach would be to revisit the current definition and normocalcemic hyperparathyroidism should be defined as having the average calcium within normal limits (and not requiring it to be persistently normal). Using this approach, PHPT should be considered as having a high average calcium. In this case, 10 out of our 11 included patients would be classified as NPHPT. This could be challenging as we cannot be sure how many measurements of calcium would be enough for this. Hypothetically, and using the results from this study, if a person was in the upper normal of the range, they would need more measurements than a person with a baseline calcium that is in the mid of the interval. This approach could be further evaluated in a research setting.

Another way forward would be to consider NPHPT as a variant of PHPT and follow up and treat according to the current guidelines. This issue has been recently addressed by the first European Society of Endocrinology Workshop (PARAT). In their consensus statement, they mentioned that the individual variation in total calcium is four times narrower than the population reference interval and the inter individual variability. I have found similar results in my study. In order to make sure that a person has normal calcium or not, one needs to know their individual range of variability. There is a chance that an individual might have an elevated calcium in comparison with their individual range, but still within the normal population reference interval for calcium. They conclude that these patients should probably be characterised as having primary hyperparathyroidism and not normocalcaemic hyperparathyroidism and be followed

up and treated accordingly. This is of course after excluding causes of secondary hyperparathyroidism (Bollerslev et al, 2019). This is supported by many studies in the literature showing similar characteristics from these two groups of patients. It is also supported by the fact that the underlying pathology of NPHPT seems to be similar to PHPT. I believe, based on the results from this study, that this might be the best approach to follow in clinical practice.

This study did not find any NPHPT patients according to the international criteria. However, I do understand that in everyday practice, physicians might evaluate patients with different characteristics than this cohort and some patients with classic NPHPT might be identified. This analysis was based on a large group and provided some lessons that can be applied to individuals in everyday practice even if the population is different. I have shown that probably the PTH reference range for older people is wrong and needs to be re-evaluated. I have also shown, using the large dataset of the UK Biobank, that probably the Pathology Harmony Range is also not the ideal to use. Patients with NPHPT can often have high values of calcium and that is normal for them and I have provided estimates of variability that can be used in everyday practice. It is crucial that all causes of secondary hyperparathyroidism have to be excluded before labelling a patient with a disorder and this can be challenging as mentioned above. Overall, I believe that NPHPT patients should be followed up and managed according to the PHPT guidelines.

5.1.2 Normocalcaemic hypoparathyroidism

5.1.1.3 Summary of findings

A pathophysiological counterpart to NPHPT is normocalcaemic hypoparathyroidism (NHYPO), which is characterised by normal levels of calcium with persistent low levels of parathyroid hormone (PTH). There is little in the current literature about this disease, with only two studies (reporting three different populations) published on its prevalence, whilst its natural history remains relatively unknown (Cusano et al, 2013a; Palermo et al, 2015). Two of these populations were then checked six and eight years later and only 0.6% and 0.09% respectively were still characterised as having NHYPO. None developed hypoparathyroidism. Serum calcium and PTH was only checked on two occasions in these studies, so the natural history of serum calcium is unclear. To my knowledge, this is the first study of the prevalence in a referral centre and the also the first study of the natural history of this disease.

I retrospectively evaluated 6280 patients attending a referral centre and identified eighteen patients (mean age 45 years, 61% female) with NHYPO on the index date (prevalence 0.29%). Out of them, approximately a third of them had intermittent hypocalcaemia. None developed hypoparathyroidism as evidenced by persistent hypocalcaemia. There was a limited number of PTH measurements throughout the patients' follow up in this study; eight patients out of the eighteen identified as having NHYPO had only one measurement available. This indicates, that probably physicians are not so concerned about finding a low level of PTH and they do not tend to follow up this further. However, this disorder could be the subclinical form of hypoparathyroidism.

5.1.1.4 Clinical relevance and challenges

Normocalcemic hypoparathyroidism is a disorder poorly characterised, with unknown consequences. When physicians are faced with the biochemical findings of low PTH and normal calcium, they usually ignore the finding and try to re-assure the patient. However, these patients might be prone to hypocalcaemic after receiving certain medication. Moreover, simple symptoms like vomiting could precipitate low calcium levels. Hypocalcaemia could be related to clinical implications like cardiac arrhythmias and epileptic seizures (Cooper & Gittoes, 2008). I believe that these patients should be monitored. Further information on this disorder is needed before any official recommendations are made but I think that NHYPO should be mentioned in any future guidelines on hypoparathyroidism.

Addressing the prevalence of NHYPO also had its challenges. As several patients have episodes of hypocalcaemia, a different number could be found according to the date of the study. I have shown, in the UK Biobank study, that patients with hypocalcaemia have the greatest variability. This could complicate the monitoring of these patients.

I have also provided estimates of the least significant change. The least significant change given, could be used in two ways in these patients. First, it gives a range to which values are expected as mentioned above for NPHPT. This can provide some reassurance for the patient and the physician. Moreover, it can be a marker of whether an individual has an actual significant change of their calcium in case of observed hypocalcaemia after an intercurrent illness or drug therapy that can cause low calcium.

5.1.3 Strengths, limitations and challenges of the metabolic bone centre studies

5.1.1.5 Using a referral centre

The main part of this study has been completed using data from a tertiary metabolic bone referral centre. This has some strengths and limitations. The subjects in this study had been evaluated for causes of secondary osteoporosis and so they might be different from the general population. On the other hand, disorders of calcium metabolism are often usually diagnosed during the evaluation of secondary osteoporosis and thus, mainly found in referral centres. In the case of NPHPT, studying a referral population has the advantage of evaluating how common NPHPT is in a referral population with osteoporosis and whether screening for NPHPT is useful.

I compared the MBC population with the UK Biobank population, which is believed to represent the wider UK general population. The UK Biobank population was younger and had a similar number of female and male participants, but these characteristics were the inclusion criteria for the study. The distribution of hypocalcaemia and hypercalcaemia was somewhat similar, with hypercalcaemia being more common in the MBC. The variability of calcium was also similar; hypocalcaemic patients were the most variable.

This was a retrospective observational study and the number of measurements varied from patient to patient. The interval between the measurements was not the same, nor was the number of measurements, as expected in a real-life setting. Obtaining data retrospectively also had challenges, like different coding used from the laboratory at different time points, making the acquisition of data not as straightforward.

Studies using real-life data have further limitations like not ideal blood sampling techniques. The patients were not fasting and a tourniquet was usually used. The methods for vitamin D and eGFR changed during the follow up period and that might have affected some results. The impact of implementing the CKD-EPI equation is that it may have improved the accuracy in patients with an eGFR of 50-60 ml/min/1.73 m^2 and might have resulted in fewer people being clustered as CKD. However, it might have increased the number of older people having CKD.

5.1.1.6 Statistical approaches

5.1.1.6.1 Mahalanobis distance for identifying outliers

Most studies on calcium disorders use the laboratory reference intervals of calcium and PTH to define NHYPO and NPHPT. I decided to use a statistical approach called Mahalanobis distance which is a robust way of identifying outliers when there is correlation between the values. As calcium and PTH are not independent, I thought this would be the best approach as it takes this correlation into account. As mentioned above, this approach also took into account the fact that PTH reference intervals are probably not ideal, as they do not take into account the fact that PTH could increase in older individuals. It has been suggested that age-related PTH reference intervals should be established (Eastell et al, 2014) and, using this approach in this study, I support this statement.

Mahalanobis distance on the other hand is specific for the population as it takes into account the available data given and establishes the centre of the cluster; it then measures how far each observation is from this centre, taking into account the shape of the cluster. Therefore, if one patient from this centre was then studied in another

centre using the same approach, they might have been identified differently (normal or abnormal respectively).

Mahalanobis distance only defines the outliers in a multi-dimensional space (in this case, two-dimensional). The next step in this study was to define the different disorders of calcium metabolism. There was a previous study in which Mahalanobis distance was used to describe the same disorders, using data from the general population. My study is the first to use Mahalanobis distance in a referral population. In order to get the different categories described above, Palermo et al used the reference interval for adjusted calcium, but for PTH, they used the geometric mean of the healthy women reference interval (Palermo et al, 2015). Using this approach, the authors probably assumed that the reference intervals for adjusted calcium were the correct ones and that the ones for PTH were not. Using the geometric mean in this population, they also avoided having "unclassified" subjects as seen in our study. However, this was a hybrid approach, as a range of values was used for one measurement, while a line was used for the other measurement. The geometric mean was also based on the measurements of young individuals.

As seen in both our ellipse and the ellipse by Palermo et al, there are patients that are "normal" as defined the ellipse but are above or below the reference interval for adjusted calcium respectively. A different approach would be to ignore reference intervals. This would mean that there would be five categories: normal, hypoparathyroidism, non-PTH induced hypercalcaemia, hyperparathyroid hypercalcaemia and secondary hyperparathyroidism as shown in Figure 5-1. Based on my findings that both NPHPT and NHYPO often have intermittent hypercalcaemia and hypocalcaemia respectively, this approach could be used if NPHPT would be considered as a variant of PHPT and NHYPO as a variant of hypoparathyroidism.

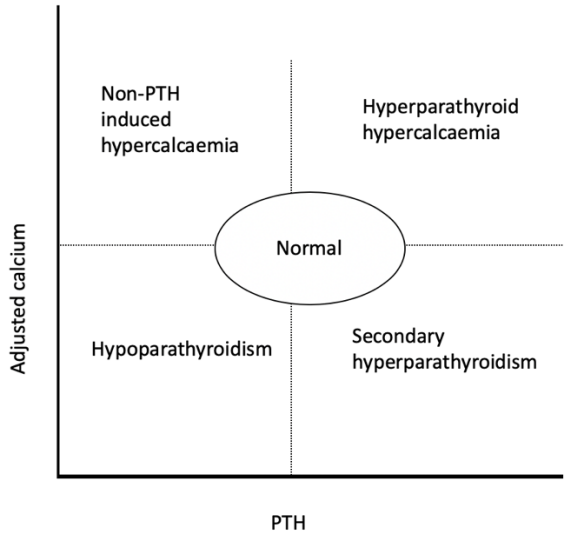


Figure 5-1: A graphical representation of the ellipse in case the geometric mean of calcium and PTH (shown in dashed lines) was used instead of the reference intervals to identify categories

I decided to use reference intervals for both calcium and PTH, to take into account not only the results of a statistical approach on outliers, but also what would be used in everyday clinical practice. If I had used Palermo's et al approach, the prevalence of both NPHPT and NHYPO would probably be higher.

By using the clinical reference intervals and the results from Mahalanobis distance, I believe that the people would be better characterised. Taking only the reference intervals has limitations. Following only the statistical approach has limitations. Taking both I removed any uncertainty and I believe that if a person is defined to be in a group, then they truly belong to that group.

5.1.1.6.2 Within subject standard deviation approach

To calculate the within-standard deviation for adjusted calcium, I used an approach described by Bland & Altman. They describe that when having multiple measurements from an individual, these measurements are generally not equal to each other and

tend to vary around a "true average". Each individual then has a standard deviation of these measurements which can easily be calculated. Since the number of measurements for each subject was different, I used the analysis of variance method to calculate the within-subject standard deviation (S_w). To follow this method, one first needs to check the assumption that the standard deviation is unrelated to the magnitude of the measurement (Bland & Altman, 1996a).

In order to perform this analysis, the assumption that standard deviations across the subjects are similar should be made. However, this is not true in all clinical situations. Using a single "mean" within-standard deviation might underestimate the variability for some patients. A suggested approach to overcome this problem is to use the 90^{th} centile of the within subject variance (Masse, 1997). I have provided these estimates in Chapter 3 and I believe that they can be used for the monitoring of patients as mentioned above.

The approach described above is usually used to calculate the so-called measurement error. In the example given by Bland & Altman, data from repeated peak expiratory flow measurements were given. These measurements were performed close to each other and the authors were trying to calculate the variability of the assay (Bland & Altman, 1996a). I used this approach to calculate the long-term variability of calcium. I therefore assumed that when taking measurements close to each other, then the pooled SD estimates the method variation and the variability of the assay. As the measurements in this population were further away, I believe that the variability is due to the within subject variability.

Another limitation of using this approach is that I am making the assumption that all patients remain stable and there is not disease progression, therefore that variations

are due to natural variation. To check this assumption, I used the history graphs of adjusted calcium over time for every subject. I have excluded any patients with progression and the estimates were similar.

This was a retrospective observational study and the number of measurements varied from patient to patient. The interval between the measurements was not the same, as expected in a real-life setting. This limited the possibility of further analyses. The analysis for the within-subject SD did not take into account the effect of time. Ideally, to give a more accurate estimate of the variance, a prospective observational study should be designed, having the same number of measurements for each patient at regular intervals. The analysis should be preferably done using a model that takes time into account. Despite the limitations, this study provided a guide to how adjusted calcium varies between the different groups described.

5.1.1.6.3 Regression to the mean

This is a statistical phenomenon where a variable is extreme when measured for the first time but then is closer to the mean when it is measured a second time. Some examples given in literature are blood pressure measurements; if an individual's blood pressure is measured and found to be high, and then measured again on a second occasion, then it's likely that it is going to be closer to normal. Moreover, patients with higher blood pressure measurements starting antihypertensive drugs will have greater reductions in blood pressure due to this phenomenon (Bland & Altman, 1994).

I understand that this could be a limitation in the studies where the variability was evaluated using only two measurements.

Section 2: UK Biobank studies

5.2.1 Summary of findings and clinical relevance

The UK Biobank study has provided some further understanding on the variability of adjusted calcium in different populations (hypocalcaemic, hypercalcaemic and normocalcaemic). I have observed that hypocalcaemic patients, regardless of their vitamin D and CKD status, are the most variable and this, as mentioned above, can complicate their monitoring. These were followed by the hypercalcaemic ones. The majority of normocalcaemic subjects kept a persistent normal value of adjusted calcium, although their mean measurement increased over time.

When performing a similar analysis in the MBC population, using just two measurements, I observed similar patterns in the two populations (hypocalcaemic patients being the most variable followed by the hypercalcaemic ones), although the characteristics of the two groups were significantly different (older and more likely to be female in the MBC population).

In both groups, the percentage of hypercalcaemic patients at follow-up was statistically higher at follow-up than baseline. For the MBC population, this is probably due to the fact that subjects with high calcium are more likely to have their calcium repeated. Moreover, in both populations, the mean calcium at follow up was greater than at baseline, with MBC having the greatest increase which could explain the greater percentage of hypercalcaemia at the first follow up.

The consistency of hypocalcaemia was statistically greater in the MBC population when analysing the whole cohort, while the other categories did not have significant differences. That is probably due to the fact that truly diseased patients are more likely to be found in the MBC population. On the contrary, the general population can have

incidental extreme values which then return to normal. This could be due to simple variability over time, or even treatment of patients found to have abnormalities at the first visit.

After studying the large dataset of the UK Biobank, the result suggests that the Pathology Harmony Range (2.20 to 2.60 mmol/L) is not the ideal one to use, although it has the benefit of being comparable between laboratories. The calculated reference range for this population was 2.25 to 2.56 mmol/L. Interestingly, the confidence intervals of the lower and upper limit did not include the current limits of 2.20 and 2.60 mmol/L.

This observation has clinical consequences. Moving the reference range to either direction, would affect the number of individuals that are labelled as having a disorder of calcium metabolism as described above.

The implications of this analysis may become more relevant when the results of a related study have been released. The calculations and results of this project will be used in an analysis trying to identify genetic loci that regulate circulating mineral parameters such as calcium, phosphate and alkaline phosphatase. Persistent abnormalities might be more closely linked to gene polymorphisms. Moreover, since UK Biobank did not provide an equation for adjusted calcium and a reference interval, the results of this analysis can be used to determine the different populations, not only in this study, but in other future UK Biobank studies.

5.2.2 Strengths and limitations

The strengths of this study include the fact that a large number of participants from throughout the UK was studied. Some of the limitations of this study include the fact

that the subjects' ages were limited between 40 to 69 years and probably the majority of them were in relatively good health and not confined to home or in care homes. Parathyroid hormone was not available to enable full assessment of the calcium metabolism. Only a maximum of two measurements were available, so the calculations are not so robust. On top of that, not all participants had two laboratory assessments available and there were differences in the characteristics of the participants as mentioned above which could have affected the results. Some of the samples were affected by dilution and although this was identified and excluded in most cases, it might have had a small impact on the overall results. Moreover, patients were not fasting, and a tourniquet was used. As UK Biobank did not give an adjusted calcium equation, one had to be calculated from the given data as mentioned in a previous study (Barth et al, 1996). Potassium measurements were not available by the UK Biobank, therefore patients with low potassium could not be excluded to precisely mirror the protocol.

Another limitation of this study was the fact that the assumptions for calculating the within-subject SD were not always met and the estimates should be treated with caution.

Section 3: Future plans

The study of normocalcaemic hyperparathyroidism and hypoparathyroidism provided some further understanding on these disorders. As mentioned above, my opinion agrees with the approach suggested by other researchers, which is to consider NPHPT as a variant of PHPT. If this approach is adopted, I do not think that a lot of research in this field is required.

I think a priority in the field of calcium metabolism is to establish the age-adjusted reference intervals for PTH and to evaluate whether the Pathology Harmony range should be used for calcium or not. When this is decided and agreed, then a further prospective study of the natural history of NPHPT could be performed, using measurements in a fasting state and at regular intervals. This would allow a better estimate of the within-subject SD and thus the least significant change. One of the limitations of my study is that the follow up was up to five years, and thus long-term changes of calcium could not be assessed. Therefore, in a future study, the patients could be followed for years and identify if they maintain the same levels of calcium, or if they at some point get persistent high levels. A similar study should be performed for NHYPO. The challenge with planning these studies would be to find patients that are suitable. Some of the issues would be the definition used, which reference range is the appropriate one to define these patients and the fact that is difficult to maintain similar conditions throughout the follow up, for example initiation of medications known to affect the calcium levels.

Furthermore, as mentioned above, we do not know much about NHYPO. Its clinical consequences, if any are unknown. Moreover, we are still unsure how often hypocalcaemia occurs. A prospective study should be designed to evaluate these

patients, with regular follow up and compare this group with patients with hypoparathyroidism and a control population.

The UK Biobank studies can continue when further data are available. Hopefully, measurements of PTH will be available at some point, to allow an assessment of the prevalence of NPHPT and NHYPO in the general population in UK. Moreover, data will be released on BMD evaluation and fracture numbers on these patients. This would allow a further estimate of the skeletal implications of NPHPT and NHYPO.

As mentioned above, the calculations and results of this project will be used in an analysis trying to identify genetic loci that regulate circulating mineral parameters such as calcium, phosphate and alkaline phosphatase. Persistent abnormalities might be more closely linked to gene polymorphisms.

Appendix

Section 1: Tables of studies on normocalcaemic hyperparathyroidism

Section 2: Intermediate analyses on NPHPT

Section 3: Bibliography

Section 1: Tables

Table 6-1: Studies on the skeletal and renal effects of normocalcaemic hyperparathyroidism - data on imaging

Publications	Recruitment	Study population (n), age (years), gender	Criteria	Baseline laboratory differences in study group	Tc99m-sestamibi	Kidney presentation at baseline	Skeletal presentation at baseline	Other
(Alyne layane pe reira et al, 2019) **Pernambuco, Brazil**	Prospectively recruited patients from an Endocrinology department in Brazil	NPHPT 25 age 63.2 ±10.7 years, 96% female	Normal calcium and high PTH, 25(OH)D >30 ng/ml, eGFR>60 ml/min/1.73m^2. Exclusions: medication (thiazides, lithium, antiresorptive agents), history of urolithiasis		NA	20% nephrolithiasis on ultrasound, NS difference in age, weight, PTH, 25(OH)D, calcium, phosphate, creatinine, urinary pH and 24hurinary calcium between the ones having kidney stones and	NA	NA

						the ones not		
(Amaral et al, 2012) **Pernambuco, Brazil**	Retrospectively evaluated 70 patients with PHPT from an endocrine centre	NPHPT **33,** age 63.67±13.83 years, 78.8% female PHPT 37 NS difference in age, BMI and gender	Normal total calcium and high PTH, 25(OH)D >30 ng/ml, GFR>60 ml/min Excluded: use of drugs (bisphosphonates, thiazides, anticonvulsants, lithium), gastrointestinal diseases with malabsorption, liver disease, hypercalciuria (urinary Ca/Cr>240mg/g Cr)	Higher 25(OH)D in NPHPT Similar levels of PTH, CTX, creatinine	NA	Nephrolithiasis 18.2% (NS difference with PHPT)	Previous history of fractures 15.2% (NS difference with PHPT)	**Higher BMD in distal radius in NPHPT** (0.54±0.15g/m2 vs 0.45±0.18, p=0.046) No statistical difference in spine and hip BMD
(Cakir et al, 2012) **Kayseri, Turkey**	Recruited patients from an endocrinology centre	NPHPT **18,** age 49.9±2.4 years, 89% female Controls 18 (matched for age, gender and BMI)	Normal total calcium and high PTH, 25(OH)D >20 ng/ml, **repeated Ca and PTH measurements 3 times with 2-week intervals**, no history of	Higher calcium, ALP and PTH in NPHPT No difference in phosphate	Performed in 17 17.6% positive 23.5% suspicious 58.5% negative	Nephrolithiasis 11.1% in NPHPT No comparisons available	Osteoporosis 47% in NPHPT No comparisons available	NA

			renal or liver diseases, no prescriptions known to affect calcium level					
(Castrillon et al, 2015) **Valladolid, Spain**	Prospective study of PHPT and NPHPT patients	NPHPT 26, age 65 (11) PHPT 16 NS difference in age, sex, BMD	Persistently normal total, adjusted and ionised calcium and high PTH on at least two occasions. Exclusion criteria: secondary elevations of PTH (unclear)	Lower levels of PTH, osteocalcin, PINP in NPHPT. Higher levels of phosphate No differences in CTX, 25(OH)D, 1,25 dihydroxyvitamin D	NA	NA	Osteoporosis 29%, NS difference from PHPT	No difference in hip and spine BMD. Radius not available
(Cusano et al, 2013a) **New York, USA**	Osteoporotic Fractures in Men (MrOS) study	NPHPT **9,** age 70.0±6 years, 0% female Controls 2224 NS differences in age, BMI	Normal total calcium and high PTH Excluded renal insufficiency, 25(OH)D≤20 ng/ml, thiazide use	NS differences in phosphate, CTX, PINP, TRAP 5b, testosterone, oestradiol, SHBG	NA	NA	NA	NS difference in lumbar spine or femoral BMD or cQCT
(Diaz-Soto et al, 2015)	Prospectively enrolled 61	NPHPT, n=41, 83%	Normal total, adjusted and	Lower levels of PTH,	NA	NA	NA	NS differences in

Valladolid, Spain	NPHPT and asymptomatic PHPT patients from their Endocrinology Service	female, mean age 63 years PHPT, n=20, 80% female, mean age 66 years (no differences in age, sex)	ionised calcium on two occasions within one year Exclusion criteria: eGFR<60 ml/min, 25(OH)D<30 ng/ml, FHH, hypercalciuria (>250mg/24h in females and >300mg/24h in men), other metabolic diseases and medications (thiazides, bisphosphonates, lithium)	bone turnover markers (ALP, PINP, osteocalcin) in NPHPT Higher levels of 25(OH)D, serum phosphate, magnesium and phosphate tubular reabsorption				BMD (spine and hip)
(Gómez-Ramírez et al, 2019) **Madrid, Spain**	Prospectively 104 enrolled all patients (77.0% female, mean age 60.5) referred for parathyroidectomy between 2015-2017 in an Endocrine Surgery	NPHPT: 45 had normal adjusted calcium, only 16 also had normal ionised calcium (15.4% of the original)	NPHPT: Normal adjusted and **ionised** calcium with high PTH in **on at least 3 occasions separated by at least 3 months).** Normal creatinine and	Similar levels of PTH, urinary calcium, ALP NPHPT had higher levels of 25(OH)D. Lower levels of PINP,	Tc-sestamibi: sensitivity in PHPT 81% vs 56.2% in NPHPT Ultrasound sensitivity in PHPT 50.6% vs 31.2% in NPHPT	History of nephrolithiasis 25%, NS difference	NA	Similar results of BMD (hip, radius and spine) in the two groups

	department in Madrid. Excluded those who had treatment, family history of PHPT	Age 60.6±11, 68% female PHPT:88 NS difference in age, gender	GFR, vitamin D> 30 ng/ml;. Excluded: malabsorption, history of gastric bypass, medications (thiazides, lithium, bisphosphonates)	osteocalcin, CTX	NS difference			
(Koumakis et al, 2013) **Paris, France**	Prospectively screened 413 consecutive patients referred to their centre. 193 fulfilled the criteria for PHPT. Evaluated the data of 60 patients who were operated and had longitudinal BMD follow up	NPHPT **39,** age 66.1±9.1, 92.3% female **41% had normal iCa** PHPT 21 (no difference in age and gender)	Normal adjusted calcium and high PTH Excluded causes of 25(OH)D<20 ng/ml, renal impairment (GFR<40 ml/min/1.73m^2), bisphosphonate , thiazides, anticonvulsants, lithium, gastrointestinal diseases related to malabsorption, liver disease. A thiazide diuretic test in those with	Higher phosphate and eGFR in NPHPT Lower CTX Similar levels of PTH, 24h urine calcium, ALP, osteocalcin, 25(OH)D	NA	History of nephrolithiasis 17.9% (NS difference with PHPT)	T score ≤-2.5 in at least one site 89.7% (vs 61.9% in PHPT, P=0.02) History of fracture 39.5% (NS difference from PHPT)	Similar results in BMD (spine, distal radius) between the two groups apart from femoral neck T score being significantly lower in NPHPT

			hypercalciuria **Oral ± iv calcium load test** (NPHPT: tCa and/or iCa increasing to supranormal values with only a minimal reduction in PTH) **41% had both iCa and tCa normal**					
(Lowe et al, 2007) **New York, USA**	Longitudinal study Referral to Metabolic Bone Disease Unit	NPHPT **37,** age 58±2 years, 95% female (29 postmenopausal, 6 premenopausal)	Elevated PTH with normal adjusted calcium Excluded cases with 25(OH)D<20 ng/ml, GFR<40 ml/min/1.73m^2, liver disease, hypercalciuria >350mg/24h, thiazide or lithium use, metabolic bone diseases	NA comparisons Phosphate, ALP, urinary calcium were normal. In 24% of patients elevated 1,25 dihydroxyvitamin D	16 tested 8 adenoma	History of nephrolithiasis 14%	Recent fragility fracture 11% History of fracture in adulthood 46% Osteoporosis 57% (19% osteoporosis at 2/3 sites, 8% osteoporosis at all three sites)	Osteoporosis **more frequent in spine** (34%) **and hip** (38%) than distal radius (28%)
(Marques et al, 2011)	Analysed records of 156	NPHPT **14,** age	At least two samples of total	Higher PTH in NPHPT	NA	**Kidney stones**	Fractures 21.4% vs	Osteoporosis more frequent

Pernambuco, Brazil	postmenopausal women who came to hospital to be screened for osteoporosis (referral)	60.6±14.8 years, 100% female Controls 142 (no difference in age, BMI, time since menopause)	calcium and PTH Excluded: 25(OH)D<30 ng/ml, GFR<40 ml/min, taking bisphosphonates, thiazides, lithium, metabolic bone diseases, gastrointestinal diseases with malabsorption, liver disease, incomplete records	group, similar levels of calcium, CTX. Lower levels of 25(OH)D in controls		**28.6%** (vs 0.7% in controls, P<0.001)	16.2% (NS difference from controls) Osteoporosis 35.7% Similar levels of BMD at hip and spine	in **spine** (28.5%) and **distal radius** (28.5%) than hip (21.4%) in NPHPT patients
(Maruani et al, 2003) **Paris, France**	178 patients referred for PHPT	NPHPT **34** Age 53±14 years, 68% female PHPT 144 (34 matched for age, gender, PTH)	Normal total calcium and high PTH, **iCa≤1.35 mmol/L, (**5 had PTH in the upper normal range), **oral calcium test** Excluded cases with: 25(OH)D<15 nmol/L, Mg deficiency <0.71 mmol/L,	Significantly lower PTH, urine calcium excretion, 1,25(OH)D than PHPT patients		Nephrolithiasis 35% Hypercalciuria 18%	Chondrocalcinosis 6% Radiographic bone demineralization 18%	27 out of 144 hypercalcaemic patients had normal tCa but elevated iCa

			creatinine >110μmol/L or GFR<50 ml/min/1.73m2, medications (bisphosphonates, Li, loop diuretics, thiazides, corticosteroids), associated diseases (progressive endocrine disorder, granulomatosis, neoplasia)					
(Pierreux et al, 2018) **Brussels, Belgium**	Retrospectively reviewed the medical records of 659 patients with PHPT and 'hyperparathyroidism of undefined origin' Excluded subjects because of active malignity, insufficient data, post-renal transplant patients,	NPHPT 25, age 62±12, 76% female PHPT 106 NS difference in age, sex, BMI, 25(OH)D	Normal adjusted calcium and elevated or inappropriately high PTH. Exclusions: 25(OH) < 20 ng/ml, eGFR < 60 ml/min, medications (loop and thiazide diuretics, bisphosphonates, denosumab, lithium), gastro-	NPHPT: Higher phosphate, eGFR, lower urinary calcium	NA	NPHPT: nephrolithiasis 36% Similar rates in PHPT	NPHPT: vertebral fractures 12%, osteopenia 56%, osteoporosis 25% Similar rates in PHPT	Similar BMD results at spine and hip

	secondary hyperparathyroidism, PHPT in the context of a MEN-1 or 2. Final number, 131		intestinal malabsorption disorders (coeliac, pancreatic, and biliary insufficiency) and hypercalciuria (urinary Ca/Cr ratio more than 240 mg/g Cr)					
(Silverberg & Bilezikian, 2003) **New York, USA**	Recruited eligible patients from referral centre	NPHPT 22; age 57±10	Normal adjusted calcium and high PTH **Confirmed on at least two occasions, eight patients also had normal iCa,** 25(OH)D>20 ng/ml. Excluded FHH, liver disease, renal disease, urinary calcium>87.5 mmol/24h, gastrointestinal disease with malabsorption, metabolic bone	No comparisons Four patients had mild hypercalciuria, seven had elevated levels of 1,25 dihydroxyvitamin D and phosphate was low in one patient	NA	14% had kidney stone The biochemical testing did not differ between the ones with and without kidney stones (calcium, PTH, urinary calcium)	45% had osteoporosis and 5% had vertebral fracture	More patients had osteoporosis at the spine (23%) and hip (27%) than the distal radius (14%)

			disease, medications (lithium, thiazide, oestrogens, loop diuretics, bisphosphonates, anticonvulsants)					
(Siprova et al, 2016) **Czech Republic**	1180 referrals to Endocrine Centre	NPHPT **187** PHPT 31	Normal total calcium and high PTH 25(OH)D≥20 ng/ml (patients with low vit D were treated and PTH had to be elevated after retested at 3 months). Excluded cases with: renal insufficiency, calcium malabsorption, hypercalciuria, medications (PPI, thiazides, lithium)	Lower PTH values	**<u>NPHPT patients:</u>** 118/187 (63%) tested ***Patients with permanent* normocalcaemia**: 6% adenoma ***Patients evolved to* hypercalcaemia:** 14% adenoma in normocalcaemic state, **73% adenoma in hypercalcaemic** state, (P=0.001)	Nephrolithiasis 4% (vs 22% in PHPT)	Reduced BMD 42% (vs 22% in PHPT)	**Less frequent symptoms (fatigue, indigestion, mood disorders, musculoskeletal pain)** in NPHPT (23.5% vs 71% in PHPT, P=<0.001) 76% another endocrine disease, mainly thyroid diseases

					PHPT patients: 31/31 tested. 68% adenoma			
(Tordjman et al, 2004) **Tel Aviv, Israel**	Retrospectively evaluated records of patients with PHPT	NPHPT **32,** age 60.5±10.5, 84% female	Normal total calcium and high PTH Secondary hyperparathyroidism excluded (impaired renal function). Three patients had low vitamin D levels, however correction did not alter PTH levels and did not unmask hypercalcaemia. Six patients with urine calcium >300mg/24h, were given thiazides without affecting PTH levels		22 tested, 73% adenoma 48% enlarged gland in ultrasound	Nephrolithiasis in 9.4%	Spine: 77% osteopenia, 46% osteoporosis Hip: 64% osteopenia, 36% osteoporosis	NA
(Traini et al, 2018) **Rome, Italy**	Retrospectively evaluated records from 731 patients who had parathyroidecto	NPHPT 154 (21%), age 59 ± 12.1, 88% female PHPT 577	Normal adjusted calcium and high PTH, 25OHD ≥ 20 ng/ml, exclusion of impaired	NPHPT: Higher phosphate, lower PTH	The sensitivity and positive predictive value of imaging studies was	NPHPT: urinary symptoms and/or kidney stones:	BMD decrease: 25.3%, NS difference from PHPT	

	my between 1998 and 2016	NS differences in age and gender	renal function, medications, malabsorption		similar. Sestamibi and ultrasound together failed in detecting all the diseased glands in 85.0% NPHPT patients and in 79.5% PHPT	16.2%, NS difference from PHPT		
(Tuna et al, 2015) **Ankara, Turkey**	Retrospectively evaluated 307 records of PHPT patients	NPHPT **23,** age 54.1±11.4, 87% female PHPT 284 (no difference in age and gender)	Normal adjusted calcium and high PTH, investigated on three consecutive occasions within 1-3 months. Excluded cases with impaired renal function, 25(OH)D≤20 ng/ml, liver failure, metabolic bone disease, medications (lithium, phenytoin, thiazide, loop diuretic), FHH (family history of hypercalcaemia,	Higher 25(OH)D, lower ALP, 24h urine calcium Similar levels of phosphate, PTH, creatinine	NA	Nephrolithiasis 15.4% (vs 19.4% in PTHP, NS difference)	Osteopenia 23.3%(vs 36.7% in PHPT) Osteoporosis 53.3% (vs 43.8% in PHPT) NS differences	

			urinary calcium <100mg/day)					

Table 6-2: Studies on the metabolic abnormalities and cardiovascular risks of normocalcaemic hyperparathyroidism

Publications	Recruitment	Study population (n), age, gender	Criteria	Glucose metabolism	Lipid profile	Other cardiovascular abnormalities	Limitations
(Beysel et al, 2019) **Ankara, Turkey**	Recruited PHPT patients from an Endocrinology Centre and controls from population-based screening programs	NPHPT: 35 PHPT: 60 Controls: 60 Matched for age and gender Similar rates of obesity, smoking	Normal adjusted calcium, high PTH on two separate occasions. 25(OH)D ≥30 ng/ml. Excluded renal insufficiency (creatinine clearance < 90 mL/ min), liver disease, significant hypercalciuria (urinary calcium > 350 mg per 24 h), thiazide, lithium, bisphosphonate use and other metabolic bone diseases	Glucose metabolism (glucose, insulin, HOMA-IR) were similar between the NPHPT and PHPT groups ($p > 0.05$) and statistically greater than controls Prevalence of diabetes was similar between the PHPT and NPHPT. No comparison with controls	Similar prevalence of dyslipidaemia in PHPT and NPHPT, higher than in controls. Elevated cholesterol, LDL and triglycerides at similar levels in PHPT and NPHPT, higher than in controls.	BMI was higher in the PHPT group Cardiovascular risk score was lower in controls, but similar between the NPHPT and PHPT groups Higher levels of both systolic and diastolic blood pressure at similar levels in PHPT and NPHPT, higher than in controls	
(Cakir et al, 2012)	Recruited patients from an endocrinology centre	NPHPT **18**, age 49.9±2.4, 89% female	Normal total calcium and high PTH, 25(OH)D >20 ng/ml, **repeated Ca and PTH measurements 3**	NS difference on insulin responses to OGTT and	NS difference in lipid profile (total cholesterol, HDL, LDL, triglycerides)	NA	

Istanbul, Turkey		Controls 18 (matched for age, gender and BMI)	**times with 2-week intervals**, no history of renal or liver diseases, no prescriptions known to affect calcium level No history of hypertension, diabetes mellitus, family history of diabetes, any medications affecting glucose or lipid metabolism	HOMA-IR [fasting insulin (μu/ml) x fasting glucose (mmol/L)/22.5]			
(Chen et al, 2015) **Fuzhou, China**	Retrospectively enrolled 940 Chinese subjects who visited the hospital for examination or treatment and had calcium and PTH measurements	NPHPT **11**, age 60.3±18.5 years, 45.5% female Controls 296 [NS differences in age, sex, BMI, Ca, glucose, lipids, 25(OH)D, creatinine]	Normal adjusted calcium and high PTH Excluded vitamin D deficiency (≤20 ng/ml), renal insufficiency (GFR<60 ml/min), thiazide use, hypercalciuria, malabsorption	NS difference in fasting glucose	NS difference in lipid profile (total cholesterol, HDL, LDL, triglycerides)	**Higher levels of SBP** (140±20.2 vs 131.2±16.5, p=0.041) and **DBP** (85.2±12.4 vs 76.8±10.3, p=0.026) in NPHPT group vs controls After adjusting for all the potential confounders, odds ratio of SBP and DBP in NPHPT were 1.035 (1.000 to 1.071) and	Not surveyed smoking, alcohol consumption, physical activity, diets

Table 2: The metabolic abnormalities and cardiovascular risks of NPHPT

						1.063 (1.004 to 1.125)	
(Diri et al, 2014) **Istanbul, Turkey**	NPHPT patients from Cakir et al 2012 study, **follow up for 4 years**	NPHPT **15**, 88% female	Definition by Cakir et al 2012 Did not include the patient progressing to hypercalcaemia	3/5 patients with IGT developed DM (overall 18.75% of patients with NHPER had DM). none of the patients with normal glucose tolerance developed glucose intolerance	Lipid levels remained at similar levels during the 4 years	NA	
(Hagstrom et al, 2006) **Uppsala, Sweden**	**Population-**based screening of 5202 **postmenopausal women** Controls 30, normal calcium and PTH (matched for age and gender, similar levels of exercise and smoking)	NPHPT **30**, mean age 66.4, 100% female	Serum Ca 2.50-2.60 (normal <2.6 mmol/L) and PTH≥35 (normal 12-55 ng/L) or ▪Ca <2.5 mmol/L and PTH>55 ng/L **Investigated 4 consecutive occasions, Normal iCa,** normal creatinine values<98 µmol/L (except one subject 122 µmol/L), no family history of hypercalcaemia, normal or high urinary excretion (excluding FHH)	**Higher levels of glucose vs controls** (5.29±2.1 mmol/L vs 4.75±1.2, P=0.007) NS difference in HbA1c	**Lower levels of HDL** (1.31±0.31 mmol/L vs 1.40±0.36, P=0.013) **Higher levels of VLDL and triglycerides** (0.62±0.63 mmol/L vs 0.528±0.46, P=0.032) and (1.93±1.1 mmol/L vs 1.72±0.88, P=0.007) **Higher LDL/HDL ratio** (3.92±1.3 vs 3.64±1.2, P=0.035)	**Higher levels of urate** (338±90 mmol/L vs 251±60, P=0.004) **Higher BMI** (28.5±4.8 kg/m^2 vs 26.1±4.3, P=0.009)	**Absence of vitamin D measurement** Some cases treated with lipid lowering therapy

Table 2: The metabolic abnormalities and cardiovascular risks of NPHPT

(Mesquita et al, 2017) **Pernambuco, Brazil**	Recruited patients with NPHPT from an endocrinology centre	NPHPT 13 66.15±7.71 Control 16 All female, matched for age	Normal adjusted calcium, high PTH. Exclusions: GFR <60 mL/min, 25(OH)D< 20 ng/ml, use of thiazide diuretic or lithium, and presence of gastrointestinal disorders associated with malabsorption	Lower fasting glucose and HbA1C in NPHPT Same rates of diabetes mellitus	Similar lipid profile (cholesterol, LDL, HDL, triglycerides) Similar rates of statin use	Lower BMI in NPHPT but similar waist circumference Similar hypertension rates	Similar smoking rates, family history of early heart disease
(Tassone et al, 2013) **Turin, Italy**	Recruited patients with NPHPT out of 357 consecutive patients with PHPT from an endocrinology centre. Recruited normocalcaemic controls from outpatients clinics; these were followed up for diseases not affecting insulin sensitivity (eg goitre)	NPHPT 23, age 58.7±11.5 Controls 23 (matched for age, gender and BMI)	High PTH and normal total and **ionised calcium in more than two separate occasions**, 25(OH)D>20 ng/ml, GFR>40 ml/min/1.73m^2. Excluded liver disease, significant hypercalciuria (urinary calcium>350mg/24h), medications (thiazides, lithium), metabolic bone diseases	NS difference in insulin sensitivity and glucose tolerance. NS difference in frequency of DM and IGT	NA	NA	Absence of PTH, 25(OH)D, ionised calcium in the control group
(Temizkan et al, 2015)	Recruited patients referred to an endocrinology	NPHPT **25,** age 48.1 (8.4). 100% female	**Serum and iCa normal, persistently high PTH on two occasions**, 25(OH)D≥20 ng/dl,	NS difference in OGTT responses	NS differences in lipid profile (cholesterol, LDL, HDL, triglycerides)	NS difference in blood pressure, uric acid, CRP, TSH	All patients female

Istanbul, Turkey	centre for high PTH, normal Ca Controls recruited from research centre, having normal PTH and 25(OH)D≥20 ng/dl	Controls 25 (matched for age, gender, vitamin D, BMI)	GFR≥60 ml/min. Excluded : hypercalciuria>400mg/d, medications (lithium, phenytoin, thiazides, loop diuretics, malabsorption (gluten enteropathy, bariatric surgery, urine calcium <50mg/d) Did not include participants with conditions that might affect glucose metabolism	and HOMA-IR No correlation between PTH and glucose metabolism markers			
(Tordjman et al, 2010) **Tel Aviv, Israel**	Retrospective study consecutive PHPT patients seen in a referral centre (Endocrinology centre in Tel Aviv)	NPHPT **32**, age 60.5±10.5, 84% female PHPT 81 (no difference in age, gender) Controls 25- to study arterial stiffness (matched to 13 NPHPT and 12 PHPT patients based on	Normal total calcium and high PTH Secondary hyperparathyroidism excluded (impaired renal function). Three patients had low vitamin D levels, however correction did not alter PTH levels and did not unmask hypercalcaemia. Six patients with urine calcium >300mg/24h, were given thiazides without affecting PTH levels	Diabetes or IFG 6% (NS difference from PHPT)	Hyperlipidaemia 10% (NS difference from PHPT)	Hypertension 20% (NS difference from PHPT) **Less frequent ischaemic heart disease (IHD) and/or cerebrovascular accident (CVA) in the NPHPT vs PHPT** (3.1% vs 24.7%, P=0.007) **NS differences in non-invasive**	IMT not evaluated Retrospective study

Table 2: The metabolic abnormalities and cardiovascular risks of NPHPT

		age, gender, cardiovascular risk factors)				**arterial stiffness parameters in the 3 groups**	
(Tuna et al, 2015) **Ankara, Turkey**	Retrospectively evaluated 307 records of PHPT patients	NPHPT **23,** age 54.1±11.4, 87% female PHPT 284 (no difference in age and gender)	Normal adjusted calcium and high PTH, investigated on three consecutive occasions within 1-3 months. Excluded cases with impaired renal function, 25(OH)D≤20 ng/ml, liver failure, metabolic bone disease, medications (lithium, phenytoin, thiazide, loop diuretic), FHH (family history of hypercalcaemia, urinary calcium <100mg/day)	Diabetes or IFG 21.7% (NS difference from PHPT)	**Higher LDL levels in NPHPT vs PHPT**(145.2±27.6mg'/dl vs 125.7±33.2, P=0.022), but with **higher TSH levels** (2.40±1.38μIU/ml vs 1.79±1.14 , P=0.011) Similar levels of triglycerides, HCL and uric acid	Hypertension 53.8% (NS difference from PHPT)	
(Yener Ozturk et al, 2015) **Istanbul, Turkey**	Patients referred for osteoporosis screening Cross-sectional	NPHPT **25,** age 52.88±11.71, 92% female PHPT 24 Healthy controls 30 (age, gender and BMI matched)	Concomitantly elevated PTH, **repeatedly normal iCa and adjusted Ca,** 25(OH)D ≥30 ng/dl (when low levels where corrected with 50.000IU for 8 weeks, PTH still remained elevated, with normal Ca) Excluded cases with: hypercalciuria>400mg/24 h, GFR<60 ml/min,, medications (lithium, phenytoin, thiazide, loop	**Higher levels of fasting glucose and higher pre-existing glucose intolerance (IGT/DM) in NPHPT** vs controls. **Similar levels in NPHPT and**	NS difference in lipid profile (except higher cholesterol level in control group); higher usage of antihyperlipidemic therapy in NPHPT and PHPT groups PTH positively correlated with triglyceride levels	NS difference for blood pressure, but **higher prevalence of pre-existing hypertension or antihypertensive medications** in the NPHPT and PHPT groups compared to controls	Small sample, cross sectional

Table 2: The metabolic abnormalities and cardiovascular risks of NPHPT

			diuretics), GI disorders, low urinary calcium (<50mg/day), FHH	**PHPT** PTH concentration was significantly higher in the IGT/DM group Positive **correlation of both calcium and PTH to fasting insulin and HOMA-IR**		**Similar prevalence of metabolic syndrome in NHPER and PHPT patients**	

Table 2: The metabolic abnormalities and cardiovascular risks of NPHPT

Table 6-3: Studies on the surgical management of normocalcaemic hyperparathyroidism

Publications	Recruitment	NPHPT population (n)	Criteria	Changes in subjects undergone surgery	Histology	Adenoma weight
(Gómez-Ramírez et al, 2019) **Madrid, Spain**	Prospectively 104 enrolled all patients (77.0% female, mean age 60.5) referred for parathyroidectomy between 2015-2017 in an Endocrine Surgery department in Madrid. Excluded those who had treatment, family history of PHPT	NPHPT: 45 had normal adjusted calcium, only 16 also had normal ionised calcium (15.4% of the original) Age 60.6±11, 68% female PHPT:88 NS difference in age, gender	NPHPT: Normal adjusted and **ionised** calcium with high PTH in **on at least 3 occasions separated by at least 3 months).** Normal creatinine and GFR, vitamin D> 30 ng/ml;. Excluded: malabsorption, history of gastric bypass, medications (thiazides, lithium, bisphosphonates)	Follow up 1 year later. There was a reduction in bone markers and improvement in BMD (no p values). Any statistical differences prior to surgery between the two groups were diminished	Incidence in multiglandular disease higher in NPHPT (7.1% vs 4.1%) but no statistical difference (p=0.84)	Weight of adenoma lower (497 ± 268 mg vs 1153 ± 1065 mg, p= 0.058)
(Koumakis et al, 2013) **Paris, France**	Prospectively screened 413 consecutive patients referred to their centre. 193 fulfilled the criteria for PHPT. Evaluated the data of 60 patients who were operated and had longitudinal BMD follow up (1 year)	NPHPT 39 **41% had normal iCa** Surgery to all patients recruited (n=39 NPHPT, n=21 PHPT)	Normal adjusted calcium and high PTH Excluded causes of 25(OH)D<20 ng/ml, renal impairment (GFR<40 ml/min/1.73m^2), bisphosphonate, thiazides, anticonvulsants, lithium, gastrointestinal diseases related to malabsorption, liver disease. A thiazide	**Significant increase of BMD in NPHPT**. Spine +2.3±5.0% (P=0.016) Hip +1.9±5.7% (P=0.048) Significant increase in BMD in PHPT patients. NS difference between groups	**Parathyroid hyperplasia or multiple adenomas more frequent** in NPHPT (28.2% vs 4.8% in PHPT, P=0.04)	**Lower adenoma weight** in NPHPT (189±181.5mg vs 354.4±296.8 in PHPT, P=0.03)

			diuretic test in those with hypercalciuria **Oral ± iv calcium load test** (NPHPT: tCa and/or iCa increasing to supranormal values with only a minimal reduction in PTH) **41% had both iCa and tCa normal**	**Significant decrease in distal 1/3 of radius BMD in NPHPT** (-1.5±3.5%, P=0.02). BMD remained stable in the radius in the PHPT group		
(Koumakis et al, 2014) **Paris, France**	Further analysis of above mentioned patients by Koumakis et al 2013 Excluded 5 lacking BMD for one of the three investigated sites	NPHPT 36 **Only 15 (42%) had normal iCa**	See Koumakis et al 2013 Considered BMD gain significant for an individual patient if it was ≥0.030g/cm^2 at any site, without any loss of BMD of ≥0.030g/cm^2 at another site during the same period of time	**Individual BMD gain in the normocalcaemic group** in 44.4% vs 73.7% of the hypercalcaemic patients, P=0.049) NS difference when comparing the gain to patients with normal or elevated iCa Pre-surgery ALP value above median: **predictive factor for BMD gain in NPHPT**	NA	NA

(Lim et al, 2017) **New York, USA**	Retrospective cohort review of all patients who underwent para-thyroidectomy from 2006–2012 (New York), n=675. Excluded if they underwent operation for secondary or tertiary hyperparathyroidism, had prior parathyroidectomy, or incomplete data, final number n=573	NPHPT 96 Normoparathyroid hypercalcaemia (NH) 72 PHPT 405 NS difference differences in age, race, history of neck irradiation, lithium usage, family history of parathyroid disease, or presence of osteoporosis and kidney stones	Not clear but included patients with eGFR<60 ml/min/1.73m^2, lithium use, 25(OH)D<30 ng/ml	NA	Multigland disease: NPHPT (43, 45%) NH (7, 10%) PHPT (36, 9%) Significant predictors for multigland disease were the NPHPT and positive family history Patients with NPHPT were over eight times as likely to have multiglandular disease (odds ratio 8.17, 95% confidence interval 4.49 to 14.83)	NA
(Lowe et al, 2007) **New York, USA**	Referral to Metabolic Bone Disease Unit	NPHPT 37 Surgery to three NPHPT patients who progressed to hypercalcaemia and to four normocalcaemic ones	Elevated PTH with normal adjusted calcium Excluded cases with 25(OH)D<20 ng/ml, GFR<40 ml/min/1.73m^2, liver disease, hypercalciuria >350mg/24h, thiazide or lithium use, metabolic bone diseases	Normalisation of calcium in the hypercalcaemic, no change in the normocalcaemic despite significant fall in PTH (returned to normal)	Hypercalcaemic patients: single adenoma (n=2), two hyperplasic, glands (n=1) Normocalcaemic: single adenoma (n=1),	Hypercalcaemic patients: single adenoma 500 mg two hyperplasic, glands weighted 100mg each Normocalcaemic: single adenoma 200mg,

					one hyperplastic gland (n=2), two hyperplastic glands (n=1)	hyperplastic glands (140-700mg)
(Maruani et al, 2003)	178 patients referred for PHPT	NPHPT 34 Surgery: 21 normocalcaemic, 73 hypercalcaemic	Normal total calcium and high PTH, **iCa≤1.35 mmol/L, (**5 had PTH in the upper normal range), **oral calcium test** Excluded cases with: 25(OH)D<15 nmol/L, Mg deficiency <0.71 mmol/L, creatinine >110µmol/L or GFR<50 ml/min/1.73m^2,taking drugs (bisphosphonates, Li, loop diuretics, thiazides, corticosteroids), associated diseases (progressive endocrine disorder, granulomatosis, neoplasia)	NA	Normocalcaemic: adenoma to all Hypercalcaemic: 65 single adenomas, 2 double adenomas, 6 hyperplasia	Parathyroid gland mass (mg), expressed in geometric mean, 95% confidence intervals Lower in NPHPT [229 (36-1464) vs 500(70-3575), p<0.05)]

			Persistence not checked at baseline			
(Pandian et al, 2019) **Boston, USA**	Evaluated data from 7569 patients who underwent parathyroidectomy from the Collaborative Endocrine Surgery Quality Improvement Program database. Excluded patients with familial, secondary or tertiary hyperparathyroidism. 733 (9.7% NPHPT)	NPHPT 733 (9.7%)	Normal calcium and elevated PTH preoperatively Note: 25.6% of patients had low vitamin D and 15.7% not recorded Kidney function not available on this database	NA	NPHPT: lower rates of single parathyroid removed (47.5%, vs 73.3%, p <0.05). Higher frequency of multigland hyperplasia in NPHPT (43.1% vs 21.9%, p < 0.05).	NA
(Sho et al, 2019) **Los Angeles, USA**	Evaluated all the patients that had parathyroidectomy between 2006-2016	NPHPT 90 (18%), 71 had a biochemical follow up>6months after surgery Median age 67 years, 89% female	Normal calcium and high PTH. Only patients with peak calcium within the reference range within 1 year prior to surgery. Excluded patients with 25(OH)D<20 nmol/L	Significant reductions on calcium and PTH in all Normal PTH after > 6 months: 53.5%, detected after a	Multigland disease: 38 of 71 patients (53.5%) Out of them, 23 (60.5%) had hyperplasia and	NA

			and eGFR<60 ml/min/1.73m^2 **10 patients had normal adjusted calcium but high ionised calcium**	median of 7.7 months **BMD improvement of 5.6% after >2 years** in patients normalising their PTH after surgery. NS change in the ones that had elevated PTH	15 (39.5%) had double adenomas	
(Silverberg & Bilezikian, 2003) **New York, USA**	Recruited eligible patients from referral centre, n=2055	NPHPT 22 (18%)	Normal adjusted calcium and high PTH **Confirmed on at least two occasions, eight patients also had normal iCa,** 25(OH)D>20 ng/ml. Excluded FHH, liver disease, renal disease, urinary calcium>87.5 mmol/24h, gastrointestinal disease with malabsorption, metabolic bone disease, medications (lithium, thiazide, oestrogens, loop diuretics, bisphosphonates, anticonvulsants)	NA	One patient that progressed to hypercalcaemia operated: two adenomas	NA
(Tordjman et al, 2004)	Retrospectively evaluated records of	NPHPT 32	Normal total calcium and high PTH	NA	Surgery to 12/32	688±541mg

Tel Aviv, Israel	patients with PHPT in a referral centre	follow up 20 surgery 12	Secondary hyperparathyroidism excluded (impaired renal function). Three patients had low vitamin D levels, however correction did not alter PTH levels and did not unmask hypercalcaemia. Six patients with urine calcium >300mg/24h, were given thiazides without affecting PTH levels		Pathology report in 11 9 single adenoma, 2 hyperplasia	
(Traini et al, 2018) **Rome, Italy**	Retrospectively evaluated records from 731 patients who had parathyroidectomy between 1998 and 2016	NPHPT 154 (21%), age 59 ± 12.1, 88% female PHPT 577 NS differences in age and gender	Normal adjusted calcium and high PTH, 25OHD ≥ 20 ng/ml, exclusion of impaired renal function, medications, malabsorption	BMD results in 12 patients, 6 (50%) had stability, 5 (41.7) had an improvement after 72.9 ± 46.8 months Ten patients with prior kidney stones before surgery had a follow up: No evidence of kidney stones in 4 (40%). One patient had persistence of microlithiasis. The rest had stable results	Multigland disease higher in NPHPT (13% vs 6.8%, $p<0.05$)	NA

(Wade et al, 2012) **Wisconsin, USA**	Retrospectively evaluated records from 771 patients who had parathyroidectomy between 1999 and 2008	58; out of them **50 had ionised calcium elevated and 8 had normal** All had surgery	Normal total calcium three months prior to surgery. Patients with recurrent, familial, secondary or tertiary hyperparathyroidism were excluded	The results of the surgery did not differ between the groups (similar number of single gland and multiple gland disease) and the intraoperative PTH measurement was successful in the same number of patients	Sestamibi and ultrasound were performed in 98%. NS difference regarding the sensitivity of positive predictive value in regards to detecting single gland disease. In patients with multigland disease, no method identified all abnormal glands	Similar gland weight: median of 360mg in the elevated iCa group vs 285 mg in the normal iCa (p 0.27)
(Yu et al, 2019) **Harbin, China**	Retrospectively evaluated 197 patients that had parathyroidectomy between 2008-2017	NPHPT 35 (18%), age 48.39±12.04, 75% female PHPT 162 NS difference in gender and age	Normal adjusted calcium and high PTH, histologically proven, 25(OH)D>30 ng/ml, GFR> 60 ml/min. Exclusions: bisphosphonates, thiazides, anticonvulsants, lithium; metabolic bone diseases or gastrointestinal diseases associated with malabsorption, liver disease	NA	NPHPT vs PHPT Adenoma: 77% vs 83% Hyperplasia: 23% vs 14% Carcinoma: 0% vs 2% p=0.108	Similar lesion diameter in cm

Table 3: Surgical management of NPHPT

Table 3: Surgical management of NPHPT

Section 2: Intermediate analyses on NPHPT

6.2.1 Analysis 1

6.2.1.1 Problems identified in Analysis 1

6.2.1.1.1 Assay change

As mentioned in the Methods section, a new method for measuring total calcium was introduced in January 2013. Moreover, there were four changes in the adjusted calcium equation during the period of this study. According to the laboratory, these changes affected the adjusted calcium results and therefore, affected patient care.

To test the effect of these changes in the population studied, a parametric test (independent Student's t-test) was performed to check whether there was any statistical difference between the means of the patients with calcium with index day before January 2013 and the patients with index day after. The mean calcium of the first group was 2.40 mmol/L (n=2390) and the mean of the second group was 2.35 mmol/L (n=4676). There was a statistically significant difference between the two groups (p value <0.001).

Since adjusted calcium is a crucial result for this study, the changes of the methods might have had an effect on the number of NPHPT patients found.

6.2.1.1.2 Mahalanobis distance calculation

Up to this point, the Mahalanobis distance analysis was not performed by myself; it was considered a complicated analysis performed in R software which I did not have the knowledge to use. By the end of the study on NPHPT, there was an attempt to reproduce the results of the Mahalanobis analysis. The results of the outlier number

were slightly different each time the analysis was attempted. The differences were due to random sampling using the estimation of the covariance matrix. The “cov.mve” function used in the R statistical software was searching the bivariate space for the minimum ellipsoid and was finding different solutions based on a random staring point. In order to be able to reproduce the same results each time the code is run, a random sampling seed has to be set in the beginning of the analysis. The problem was that the initial seed was not known and that is why the results would not be able to be reproduced again.

6.2.1.1.3 Problems with database

There was a column with the age on the day of the scan, but it only included the ages of subjects still alive on the day of the database acquisition, so there were data missing. The database only included results for adjusted calcium as given by the laboratory and did not have any measurements of total calcium and albumin to allow manual calculations of adjusted calcium.

6.2.1.2 The decision to address the problems

Since the previous analysis was not reproducible and the results were probably affected by the change in the calcium measurement and the adjusted calcium equation, a new approach was attempted using updated patient data.

6.2.1.3 Databases received before analysis 2 and their problems

In order to run the updated analysis, a new database containing patients referred for a BMD until 2017 was requested and retrieved. All of them had laboratory work-up performed within 28 days from their scan. In the previous analysis, several of the subjects did not have results on both calcium and PTH and that is why there were not included. To address that issue, the subjects in the updated database had to have both calcium and PTH available. Moreover, in the original database, only adjusted calcium was included; total calcium and albumin were also requested in the subsequent databases, to allow the calculation of adjusted calcium.

There were several different databases received and checked before the analysis. The issues with each one are described below. A summary of all the received databases can be also be seen in Table 6-7. For each database received, there was an initial check to make sure that there not any issues.

- *Database "SearchServiceResultsSTH15691_AllUpdated*

It contained data for 12623 patients attending the Metabolic Bone Centre for a BMD from 01/12/2011 to 10/1/2017.

Problems: Out of the 12623 patients, 6062 had both adjusted calcium and PTH compared to 5977 in the old one, therefore, it only contained 85 new patients compared to the previous one

Reason: The code for PTH had changed, so the patients with updated PTH codes could not be identified

- *Database "SearchServiceResultsSTH15691_WithPTHCorrected*

It contained data for 7885 patients attending the MBC for a BMD from 01/12/2011 to 31/1/2017. The patients had to have both adjusted calcium and PTH available to be included.

Problems: The referring clinician was not included

- *Database "SearchServiceResultsSTH15691_WithLabsRequestingClinician"*

Same database as before but with name of the consultant who requested the test available.

Problems: The dates for adjusted calcium, calcium and albumin should be the same, since the laboratory used the calcium and albumin values to calculate the adjusted calcium. There were differences in these dates, e.g. a patient had adjusted calcium in 2012 and calcium in 2014. Moreover, date of birth (DOB) was not available and some patients did not have their age recorded

Reason: The extractor was looking for the first available value for the DXA scan columns, so in some instances when there was no Z-score available on their first scan, it looked for the next result which would be from a later scan. Thus, when the date for the Z-score was different to the T-score, some laboratory columns were referencing one or the other.

To get the next database, all the laboratory columns referenced the first T-score column (lumbar spine). As a result, some patients did not have Z-scores at the same day.

- *Database "SearchServiceResultsSTH15691_WithDOB"*

Contained data form 7792 patients. Some patients were not included because of the change in the extractor script code.

Appendix: Intermediate analyses on NPHPT
Analysis 1

Problems: The calculated age on the day of the scan (= day of scan – date of birth) and the given age on the database were not always the same.

Moreover, there were a lot of eGFR measurements missing (72.9%), although creatinine measurements were available. Since the eGFR value is included in the definition on NPHPT, the missing data would affect the results.

Reason: The age column given was calculated when the data were loaded into the system and was not accurate, so I was advised to stop using it. The reason there were so many eGFR values missing was because the laboratory changed the code used in the system and both the old and new code had to be included in the extractor code.

- *Database "SearchServiceResultsSTH15691_WithAllEGFRCodes"*

Contained 7809 patients attending the MBC between 01/12/2011 and 31/1/2017 for a DXA scan. Only the ones having adjusted calcium and PTH available were included. This database was used for the analysis 2 described below.

6.2.2 Analysis 2 (completed in 2017)

6.2.2.1 Preparation of database

The steps described below were followed for this analysis. The software used in general was Microsoft Excel, while R studio was used for the Mahalanobis distance analysis. As mentioned above, this database contained 7809 patients attending the MBC between 01/12/2011 and 31/1/2017.

- Add study numbers A1 to A7809
- Calculate age on day of scan

  (Date when they had lumbar T score- date birth)/365

- Calculate (recorded in one decimal place) and check BMI as mentioned above
- PTH columns. In May 2015 there was a change in the PTH units and so the updated databases included subjects with PTH in ng/L and others with PTH in pmol/L. Two extra columns were added containing PTH in ng/L and pmol/L for all the patients. The results in pmol/L had to be multiplied by 9.43 to be transformed into ng/L and were recorded with zero decimal places. The results in ng/L were multiplied by 0.106 to be transformed to pmol/L and were rounded to one decimal place. There were fourteen patients with no PTH available, because instead of a value they had the clinician's name recorded. There also two patients with no calcium available. All these patients were excluded from the analysis. The number of patients described from this point forward was 7793. The ng/L values were used and extra columns defining high, normal and low PTH were added
  - Low PTH (<15 ng/L), n=39
  - Normal PTH [15-65 ng/L], n=5981

  - High PTH (>65 ng/L), n= 1773

- Calcium, adjusted calcium and albumin. The new method for measuring calcium was introduced on the 14th January 2013.
  - Calcium measurement before the change, n=2080
  - Calcium measurement after the change, n=5713

  Only the 5713 patients with the new method calcium were used for the next steps. Moreover, from September 2013 onwards, there was a change in the adjusted calcium equation. Out of the 5713 patients included, 3064 had their equation calculated with the previous method. The new equation was used to calculate adjusted calcium for all the patients

$$Adjusted\ Ca = Total\ Ca + [0.0172(43 - Albumin)]$$

  As a result, 172 patients had low levels of adjusted calcium (<2.20 mmol/L) and 233 had high levels (>2.60 mmol/L).

- 25(OH)D levels. Out of the 5713 patients, 2325 (41%) had low levels (<50 nmol/L) and 2784 had normal (≥50 nmol/L). The rest (n=604) did not have any result available.
- eGFR levels. Out of the 5713 patients, 1045 (18%) had low levels (<60 mol/min/1.73 m^2) and 4565 had normal (≥60 mol/min/1.73 m^2). The rest (n=103) did not have any result available.
- Save file with only new method calcium patients and with both PTH and adjusted calcium available. **Number of patients to analyse: 5713. Patient characteristics: Mean age 66 years, 72% female.**

6.2.2.1.1 Normocalcaemic hyperparathyroidism patients

Based on the laboratory results on the index day, 226 patients being outside the ellipse and having normal adjusted calcium and high PTH, were identified. Using the international criteria on NPHPT, 129 patients were excluded because of either low eGFR or no eGFR measurement. Moreover, 76 patients with normal eGFR, were excluded because of 25(OH)D<50nmol/L (n=67) or no vitamin D measurement (n=9) (Table 6-4). This resulted in identifying 21 patients, with likely NPHPT (Table 6-5).

		25(OH)D (nmol/L)		
		≥50	<50	NA
eGFR (ml/min/1.73m^2)	≥60	**21**	67	9
	<60	39	76	9
	NA	0	0	5

Table 6-4: Data for the 226 patients having normal adjusted calcium and high PTH, but being outside the ellipse (analysis 2).

After applying the cut-off for 25(OH)D<50nmol/L and/or eGFR<60ml/min/1.73m^2. 265 patients were excluded from having NPHPT. Only 21 of the 226 were left for further evaluation (seen in bold). NA: not available

	Frequency	Percentage
Normal	5080	88.9
Hyperparathyroid hypercalcaemia (HH)	160	2.8
Hypoparathyroidism	1	0.0
Secondary hyperparathyroidism	254	4.4

Non-PTH hypercalcaemia	6	0.1
Normocalcaemic hyperparathyroidism (NPHPT)	21	0.4
Normocalcaemic hypoparathyroidism (NHYPO)	22	0.4
Normoparathyroid hypercalcaemia	67	1.2
Normoparathyroid hypocalcaemia	43	0.8
Unclassified abnormal	59	1.0
Total	**5713**	**100.0**

Table 6-5: The different patient categories based on their calcium metabolism disorders, after applying the criteria for 25(OH)D<50nmol/L and/or eGFR<60 ml/min/1.73m^2

A further evaluation of their medical notes, excluded fifteen patients due to other exclusion criteria (Table 6-6). The number of NPHPT patients identified after applying the exclusion criteria in our referral population was six.

	Study number	**Analysis 2** **Include/ Exclude**	**Analysis 1 results**	**Comments**
1	A303	Exclude Sodium valproate Only one PTH available		
2	A664	Exclude Treated pseudohypoparathyroidism	6177: Excluded – treated pseudohypoparathyroidism	
3	A843	Exclude Tertiary hyperparathyroidism Renal transplant		
4	A1023	Include	3831: Excluded – just one measurement of PTH	Although excluded in previous analysis, more

			available, on bisphosphonates	abnormal results of PTH were available during the second analysis. The patient had stopped risedronate after first analysis
5	A1114	Exclude Secondary hyperparathyroidism (low GFR)		
6	A1287	Exclude Only one PTH On bisphosphonates		
7	A1349	Exclude Inconsistency of PTH	6181: Excluded – just one measurement of PTH	
8	A1742	Exclude On bisphosphonates Borderline eGFR		
9	A2308	Include		
10	A2487	Include		
11	A3329	Exclude No persistence of high PTH (only one high result)	1977: Excluded – no persistence of high PTH (only one high result)	
12	A3656	Exclude Crohn's Bisphosphonates-furosemide		
13	A3732	Exclude Just one measurement of PTH	3584: Excluded – just one measurement of PTH and calcium available	

14	A4137	Exclude Inconsistency of PTH		
15	A5113	Exclude Only one abnormal result of PTH with normal VitD (chronic vitamin D deficiency)		
16	A5137	Exclude Bisphosphonates	8483: Excluded – bisphosphonates	
17	A5179	Include		
18	A5754	Include	1849: Included	
19	A6009	Exclude Only one abnormal result of PTH with normal VitD (chronic vitamin D deficiency)	6715: Excluded – just one measurement of PTH and calcium available	
20	A6344	Exclude Renal transplant		
21	A6855	Include		Although this patient had coeliac disease, she had normal vitamin D levels and urine calcium without taking supplements, which means calcium absorption was not affected

Table 6-6: Exclusion criteria for patients identified as likely NPHPT. Fifteen patients out of 21 were excluded. The table also summarises whether these patients were identified in the previous analysis (analysis 1)

6.2.2.1.2 Conclusions

The prevalence of NPHPT in the Metabolic Bone Centre referral population using analysis 2, was 0.10% (six patients out of 5713). Their mean age was 72 years. Out of them, 83% were female.

6.2.2.2 Problems identified in Analysis 2

The patients identified up to this point, were found because they had an available T-score at the spine. However, some of the patients attending the Metabolic Bone Centre for a BMD did not have available spine measurements, due to degenerative changes at the spine, multiple vertebral fractures etc. In this case a hip BMD measurement would be the only available. Moreover, patients with bilateral hip replacements would not have BMD measurements at the hip and the only available BMD measurement could be the one at the forearm. By including patients based on the spine BMD, some of the patients attending the department would be excluded and that could lead to underestimation of the prevalence.

For that reason, another database was requested, including all the patients with results on spine, hip or forearm BMD measurement and the analysis was repeated as described below.

6.2.2.3 Databases received before analysis 3 and their problems

In order to run the updated analysis, a new database containing patients referred for a BMD until 2017 was requested and retrieved. Once again, there were several different databases received and checked before the analysis. The issues with each

one are described below. A summary of all the received databases can be also be seen in Table 6-7. For each database received, there was an initial check to make sure that there not any issues.

- *Database "SearchServiceResultsSTH15691_NonSpineDXAPatients"*

This contained data on 111 patients who had a scan between December 2011 and June 2017 and their results did not include a spine DXA.

Problems: Urine results did not correspond to the BMD results and not all 24-hour urine collections for calcium also had creatinine available. The reason was that the extractor was giving the first available urine calcium

- *Database "SearchServiceResultsSTH15691_NonSpineDXAPatientsCorrected" and Database "SearchServiceResultsSTH15691_SpineDXAPatients"*

  These included data on 113 and 8244 patients respectively that had scans between December 2011 and July 2017. It was difficult to work on two separate databases, so a new one was received.

- *Database "SearchServiceResultsSTH15691_AllDXAPatients"*

  Contained information on 8411 patients attending the MBC between 01/12/2011 and 27/7/2017 for a DXA scan at either the spine, hip (neck T-score) or forearm and a laboratory. Only the ones having adjusted calcium and PTH available were included. This is the database used for the following analyses

Appendix: Intermediate analyses on NPHPT
Analysis 2

Name of database	Date run Number of patients	First date Last date	Patients included	Problems	Reasons/Requested changes	Notes
0a. Original **Used in** Analysis 1 **(5 NPHPT patients)**	01/09/2015 9224	01/12/2011 29/05/2015	All patients attending MBC			Study numbers: 1 to 9224
0b. FromAugust2015	21/03/2016 2167	03/08/2015 22/02/2016	All patients attending MBC after August 2015			Did not use this one
1. AllUpdated	31/01/2017 12623	01/12/2011 10/01/2017	All patients attending MBC	Only 85 new patients to analyse compared to previous one	Reason: Laboratory changed the code for PTH after changing units, so patients with new codes could not be identified	Did not use this one
2. WithPTHCorrected	22/01/2017 7885	01/12/2011 31/01/2017	Only patients with both adjusted calcium AND PTH		Consultant who requested labs would be useful to have	Study numbers: 1 to 7885
3. WithLabsRequestingClinician	16/03/2017 7885	01/12/2011 31/01/2017	Same as above	Dates for adjusted calcium and calcium not the same. PTH referring clinician missing. DOB was	Reason: The extractor was looking for the first available value for the DXA scan columns, so in some instances when there was no Z-score available on their first scan, it looked for the next result which would be from a later scan. Thus, when the date for	Study numbers: 1 to 7885

				missing and some patients did not have age recorded	the Z-score was different to the T-score, some laboratory columns were referencing one or the other. Date of birth requested	
4. WithDOB	24/03/2017 7792	01/12/2011 31/01/2017	Same as above	Calculated age and provided age are not the same. A lot of eGFR values are missing although creatinine values are available	Reason: The age column given was calculated when the data were loaded into the system and is not accurate, so I was advised to stop using it. The laboratory changed the code for eGFR, so patients with new codes were not identified	Study numbers: 1 to 7792
5. WithAllEGFRCodes **Used in** Analysis 2 (**6 NPHPT patients)**	30/03/2017 7809	01/12/2011 31/01/2017	Same as above		The databases contained spine DXA. Patients not having a spine result were missed	Study numbers: A1 to A7809. Excluded patients with old measurement of calcium
6. NonSpineDXAPatients	21/07/2017 111	01/12/2011 09/06/2017	Same as above Patients with no spine result available	Urine calcium and BMD values are on different dates Many have urine calcium but not urine creatinine	Reason: It was giving the first available urine calcium	Did not use this one
7. NonSpineDXAPatientsCorrected	03/08/2017 113	01/12/2011 09/06/2017	Same as above		A single database would be more useful	Did not use this one

8. SpineDXAPatients	08/08/2017 8244	01/12/2011 29/06/2017	Patients with spine result available			Did not use this one
9. AllDXAPatients **Used in** Analysis 3 **(8 NPHPT patients)**	16/08/2017 8411	01/12/2011 27/07/2017	Contains spine, hip, forearm results			Study numbers: C1 to C8411. Changed to S1- S6280 after excluding patients with old calcium measurement

Table 6-7: Information on the databases received and used throughout the study of prevalence of Normocalcaemic hyperparathyroidism (NPHPT)

6.2.3 Analysis 3 (completed in 2017)

6.2.3.1 Preparation of database

The steps described below were followed for this analysis.

- Add study numbers C1 to C8411
- Calculate age on day of scan
    - For patients with BMD at the lumbar spine (n=8284)

      (Date of lumbar T score - date of birth)/365

    - For other patients (n=127)

      (Date of T score neck hip - date of birth)/365, (n=118)

      (Date of T score forearm – date of birth)/365, (n=9)
- Problems with filters and decimal places

  To define whether the measurements of PTH, calculated adjusted calcium, 25(OH)D and eGFR were high, normal or low, the filter function was used in Microsoft Excel.

  When calculations for adjusted calcium and PTH (conversion of units) had to be performed, the numbers had to be shown in certain decimal places. For example, adjusted calcium had to be rounded to two decimal places; a value of 2.598 mmol/L (value A) would be shown as 2.60 mmol/L and so would a value of 2.604 mmol/L (value B). Since the upper normal level of calcium is 2.60 mmol/L, some values shown as 2.60 were considered as normal (value A), and some were considered high (value B). That is because Excel showed only two decimal places, but kept the whole number in the system. That caused misclassification of patients and confusion.

For that reason, another column was formed with the calculated values copied and rounded using the *ROUND (name of column, number of decimal places) function*. This column was then used to define the level of measurement (low, normal, high) using the filter function in Excel.

- Calcium, adjusted calcium and albumin. The new method for measuring calcium was introduced in January 2013
    - Calcium measurement before the change, n= 2118
    - Calcium measurement after the change, n=6293

    Only the 6293 patients with the new method calcium were used for the next steps. Moreover, from 2013 onward, there was a change in the adjusted calcium equation. Out of the 6293 patients included, 3101 had their equation calculated with the previous method. The new equation was used to calculate adjusted calcium for all the patients

$$Adjusted\ Ca = Total\ Ca + [0.0172(43 - Albumin)]$$

    As a result, 203 patients had low levels of adjusted calcium (<2.20 mmol/L) and 260 had high levels (>2.60 mmol/L).

    The mean given adjusted calcium for the patients with the previous equation was 2.35 mmol/L, while their mean calculated adjusted calcium was 2.38 mmol/L. The calculated adjusted calcium was higher from their given adjusted calcium, by a mean of 0.03 mmol/L, as described by the laboratory. For patients with the new equation, both the mean of the given and calculated adjusted calcium were 2.37 mmol/L.

- PTH columns. Two extra columns were added containing PTH in ng/L and pmol/L for all the patients. The results in pmol/L had to be multiplied by 9.43 to be transformed into ng/L and were recorded with zero decimal places with the ROUND function. The results in ng/L were multiplied by 0.106 to be transformed to pmol/L and were rounded to one decimal place. There were 13 patients with no PTH available because instead of a value they had the clinician's name recorded. Twenty-nine patients had a low PTH (<15 ng/L), 4850 had a normal one and 1401 had a high level (>65 ng/L).
- Calculate (recorded in one decimal place) and check BMI as mentioned above. There were some patients with extreme BMIs (>100kg/m^2, n=33). For some of them, it was obvious that the height and weight had been reversely recorded in the database, and so they were corrected automatically. There were two patients with BMI>100kg/m^2. The next step was to check the form completed by the technician on the day of the BMD measurement; subject C2128 had a BMI of 142.7 kg/m^2 (height was 70.1cm, weight 70.1kg). Correct height was 151.7cm. Subject C4365 had a BMI of 1800.0 kg/m^2 (height 15 cm, weight 40.5 kg). Correct height was 151 cm. There were also patients with BMI<5 kg/m^2 (n=10). This was because weight was not available
- Save file with only new method calcium patients and with both PTH and adjusted calcium available. The thirteen patients without a PTH available were excluded. Total number of patients, n=6280
    - 25(OH)D levels. Out of the 6280 patients, 2531 (40%) had low levels (<50 nmol/L) and 3038 had normal (≥50 nmol/L). The rest (n=711) did not have any result available. Therefore, from the subjects that had a measurement available, 45% had low vitamin D

- eGFR levels**.** Out of the 6280 patients, 1137 (18%) had low levels (<60 mol/min/1.73 m^2) and 5035 had normal (≥60 mol/min/1.73 m^2). The rest (n=108) did not have any result available. The numbers according to different CKD stages can be seen in Table 6-8.
- Number of patients to analyse: 6280. Mean age 66 years (range 16-100 years), 72% female (n=4527), 40% vitamin D deficient

- Add new study numbers: S1 to S6280

Category	**GFR ml/min/1.73 m^2**	**Number of patients**
G1	≥90	2232
G2	60-89	2803
G3a	45-59	733
G3b	30-44	311
G4	15-29	72
G5	<15	21
Not available		108

Table 6-8: Distribution of the 6280 patients that were included in the analysis into chronic kidney disease stages

6.2.3.1.1 Mahalanobis distance, the different ellipses

The Mahalanobis distance analysis was performed to define the outliers and divide the patients into different categories. **The ellipse in Figure 6-1 is also included in the final manuscript in Chapter 3**.

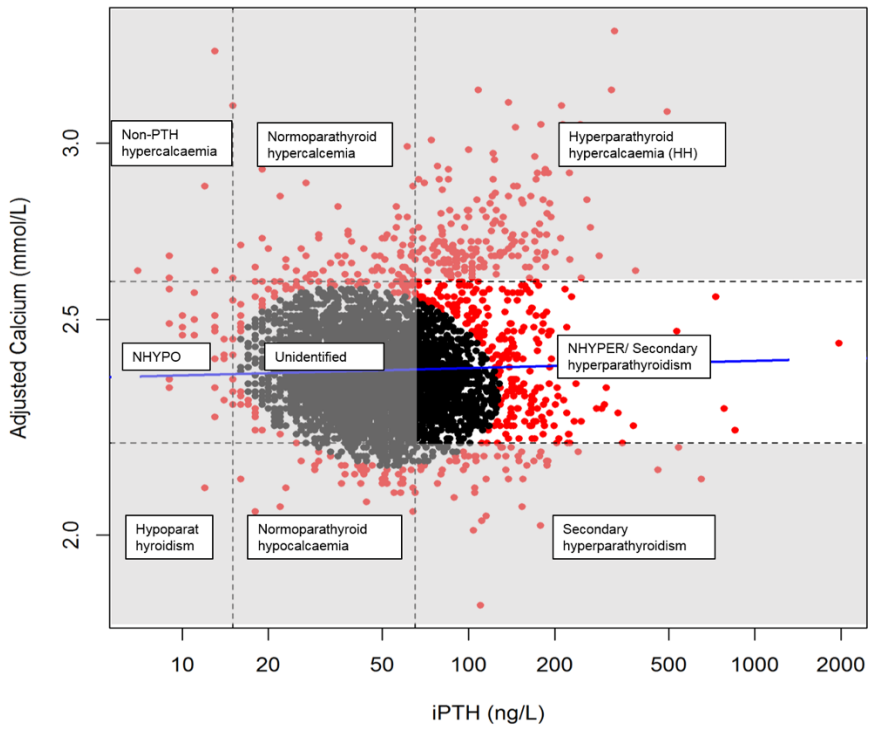


Figure 6-1: Data results from adjusted calcium and PTH.

The ellipse was formed using a statistical method (Mahalanobis distance) to identify "normal" subjects (black dots) and "abnormal" ones (red dots). The reference range of both adjusted calcium and PTH (horizontal and vertical dashed lines respectively), were used to identify patient categories as seen above. The white area includes patients with normal adjusted calcium and high PTH (n=265); these were either potentially NPHPT patients [given that 25(OH)D≥50nmol/L and eGFR≥60 ml/min/1.73m^2] or secondary hyperparathyroidism patients [given that 25(OH)D<50nmol/L and/or eGFR<60 ml/min/1.73m^2]. The Pearson correlation coefficient was 0.034 (95% CI: 0.009 to 0.058, p value 0.007). The regression line is indicated in blue.

6.2.3.1.2 Normocalcaemic hyperparathyroidism patients

Based on laboratory results on the index day, 265 patients being outside the ellipse and having normal adjusted calcium and high PTH, were identified. Using the international criteria on NPHPT, 145 patients were excluded because of either low

eGFR (<60 ml/min/1.73m^2) or no eGFR measurement. Moreover, 82 patients with normal eGFR, were excluded because of 25(OH)D<50 nmol/L (n=78) or no vitamin D measurement (n=14) (Table 6-9). This resulted in identifying 28 patients, with likely NPHPT. The rest of the patient categories can be seen in Table 6-10.

		25(OH)D (nmol/L)		
		≥50	<50	NA
eGFR (ml/min/1.73m^2)	≥60	**28**	78	14
	<60	46	83	11
	NA	0	0	5

Table 6-9: Data for the 265 patients having normal adjusted calcium and high PTH (analysis 3).

After applying the cut-off for 25(OH)D<50nmol/L and/or eGFR<60ml/min/1.73m^2, 237 patients were excluded from having NPHPT. Only 28 of the 265 were left for further evaluation (seen in bold). NA: not available

Categories	**Number (%)**
Normal	5574 (88.76)
Hyperparathyroid hypercalcemia (HH)	172 (2.74)
Hypoparathyroidism	1 (0.02)
Secondary hyperparathyroidism	291 (4.63)
Non-PTH hypercalcemia	6 (0.10)
Normocalcemic hyperparathyroidism (NPHPT)	28 (0.45)
Normocalcemic hypoparathyroidism (NHYPO)	22 (0.35)
Normoparathyroid hypercalcemia	67 (1.07)
Normoparathyroid hypocalcemia	43 (0.68)
Unclassified abnormal	76 (1.21)

Table 6-10: The different patient categories based on their calcium metabolism disorders

Note: The results of analysis 3 up to this point, are the ones that are also included in the final analysis (Chapter 3) and were not further altered. The final code used to calculate Mahalanobis distance is included in the Supplementary material (code 1.1).

The process of selecting the final NPHPT patients was challenging and was updated. The thought process and intermediate results are described below.

6.2.3.1.2.1 Selecting the final normocalcaemic hyperparathyroidism patients

A further evaluation of the likely NPHPT patients' medical notes and their follow up laboratory investigations from 2011 to the end of 2017, excluded twenty patients (Table 6-11). Once again, it was obvious that there were two patterns of calcium variations in these patients; some patients had persistent normocalcaemia throughout their follow up and some had intermittent hypercalcaemia (Table 6-12).

6.2.3.1.3 Conclusions

In total, eight patients out of a population of 6280, were identified as having NPHPT based on the index day, without checking the persistence of their laboratory evaluations (adjusted calcium and PTH). Their mean age was 70 years and seven were female. The prevalence of NPHPT using data from the index date was 0.13%. Once again, two patterns of adjusted calcium variations were identified. The majority of patients had intermittent hypercalcaemia (six out of eight). Only two patients (0.03% of the whole population) had persistent normocalcaemia throughout the follow up period (2011 to 2017).

Study number	Index date	Age	Gender	Result in analysis 3	Result in analysis 2	Result in analysis 1	Comments
S189	28/08/2014	65	F	Excluded - sodium valproate - only one PTH available	A303: Excluded - sodium valproate -only one PTH available	Not identified	
S227	01/03/2017	59	F	Excluded - probably chronic vitamin D deficiency	Not identified	Not identified	17/6/11: high PTH, low vitD, 29/11/11: normal PTH, normal vitD, 26/1/12: normal PTH, ?vitD, 16/2/12: high PTH, ?vitD, 5/12/16:? PTH, low vitD, **1/3/17: high PTH, normal vitD, probably high PTH from recent vitamin D deficiency (4m before)**
S449	02/09/2014	65	F	Excluded - treated pseudohypoparathyroidism	A664: Excluded - treated pseudohypoparathyroidism	6177: Excluded – treated pseudohypoparathyroidism	
S567	04/09/2015	72	F	Excluded - tertiary hyperparathyroidism-renal transplant	A843: Excluded - tertiary hyperparathyroidism-renal transplant	Not identified	

S696	09/01/2015	75	F	Included	A1023: Included	3831: Excluded – just one measurement of PTH available, on bisphosphonates	Laboratory investigations because of accelerated bone loss **9/1/15: high PTH, normal vitD, on risedronate since 2003. Stopped on index day** 17/1/17: high PTH, normal vitD
S757	04/09/2013	68	F	Excluded - secondary hyperparathyroidism (low eGFR)	A1114: Excluded - secondary hyperparathyroidism (low GFR)	Not identified	eGFR always< 60 apart from index day (62)
S871	21/07/2015	83	M	Excluded - only one PTH (deceased)-on bisphosphonates	A1287: Excluded - only one PTH (deceased)-on bisphosphonates	Not identified	**21/7/15: high PTH, normal vitD, on alendronate since 2011**
S911	04/09/2014	70	F	Excluded - inconsistency of PTH	A1349: Excluded - inconsistency of PTH	6181: Excluded – just one measurement of PTH	**4/9/14: high PTH, normal vitD,** 16/10/14: high PTH, ? vitD (not enough time between this and previous measurement) 29/5/15: normal PTH, started zol 2014, 5/11/15: high PTH, normal vitD, on zol,

							22/9/17: normal PTH, normal vitD, ? zol
S1194	08/01/2016	74	F	Excluded - on bisphosphonates-borderline GFR	A1742: Excluded - on bisphosphonates-borderline GFR	Not identified	Borderline eGFR (29-67), on risedronate from 2011-2016
S1620	02/12/2015	75	F	Included	A2308: Included	Not identified	Laboratory investigation: low BMD for age
S1692	10/03/2013	79	F	Excluded - bisphosphonates (zoledronic acid) on index day	A2409: Identified as HH	1236: Excluded – bisphosphonates (zoledronic acid) on index day	Oral bisphosphonates 2004-2008, Zoledronic acid 2009-2013. Measurements of PTH: 4/11/08, 7/1/09, 12/8/09, **11/3/13 -** all high**,** 7/4/16: high PTH, normal vitD,? zol, 18/7/17: high PTH, ?vitD, ?zol
S1753	21/07/2016	86	M	Included	A2487: Included	Not identified	Laboratory investigation: PHPT workup
S2406	28/10/2014	67	F	Excluded - no persistence of high PTH (only one high result)	A3329: Excluded - no persistence of high PTH (only one high result)	1977: Excluded – no persistence of high PTH (only one high result)	16/1/14: normal PTH, ?vitD, **28/10/14: high PTH, normal vitD,** 4/11/16: normal PTH, normal vitD, 19/12/16: normal PTH, normal vitD

S2453	11/03/2014	57	F	Included	A3400: Identified as HH	4777: Included	Laboratory investigation because of low BMD for age
S2654	17/07/2015	88	F	Excluded - Crohn's- bisphosphonates- furosemide	A3656: Excluded - Crohn's- bisphosphonates- furosemide	Not identified	**17/5/15: received 5 zoledronate infusions**
S2720	12/12/2013	83	F	Excluded - just one measurement of PTH	A3732: Excluded - just one measurement of PTH	3584: Excluded – just one measurement of PTH and calcium available	Only one measurement on index day
S3021	07/08/2015	64	F	Excluded - inconsistency of PTH	A4137: Excluded - inconsistency of PTH	Not identified	**7/8/15: high PTH, normal vitD,** 10/11/15: normal PTH, ?vitD, 20/5/16: normal PTH, ?vitD, 27/4/17: high PTH, normal vitD
S3703	11/05/2017	72	F	Excluded- on carbamazepine	Not identified	Not identified	2 measurements available 3/17, 5/17, both on carbamazepine for trigeminal neuralgia
S3812	11/04/2014	70	F	Included	A5105: Identified as HH	5489: Included	Laboratory investigation as a follow up to endocrine clinic
S3820	14/07/2015	49	F	Excluded - Inconsistency of PTH	A5113: Excluded - Inconsistency of PTH	Not identified	**14/7/15: high PTH, normal vitD,** 6/10/16: normal PTH, ? vitD,

							1/3/17: high PTH, low vit D
S3841	11/09/2014	84	F	Excluded - bisphosphonates (aledronic acid) on index day - PTH high after	A5137: Excluded - bisphosphonates (aledronic acid) on index day	8483. Excluded – bisphosphonates (aledronic acid) on index day	14/9/09: normal PTH, **11/9/14: high PTH, normal vitD, on alendronate since 2009,** 5/10/15: high PTH, ? alendronate
S3882	30/11/2015	69	F	Included	A5179: Included	Not identified	Laboratory investigation because of vertebral fractures
S4392	28/01/2014	66	F	Included	A5754: Included	1849: Included	Laboratory investigation as follow up to clinic appointment
S4618	07/07/2014	59	F	Excluded - only one abnormal result of PTH with normal VitD (chronic vitamin D deficiency)	A6009: Excluded - only one abnormal result of PTH with normal VitD (chronic vitamin D deficiency)	6715: Excluded – just one measurement of PTH and calcium available	16/6/14: ? PTH, low vitD, **7/7/14: high PTH, borderline normal vitD (probably due to recent vitD deficiency),** 29/3/17: high PTH, low vitD, 19/7/17: high PTH, normal vitD (only 4 months after previous, probably PTH high due to recent vitD deficiency)
S4903	02/11/2015	59	M	Excluded - renal transplant	A6344: Excluded - renal transplant	Not identified	Renal transplant

S5321	07/07/2014	78	F	Excluded – bisphosphonates (aledronic acid) on index day	Not identified	4403: Excluded – bisphosphonates (aledronic acid) on index day	4/7/14: normal PTH, normal vitD, alendronate recently started, **21/7/14: high PTH, normal vitD, alendronate recently started,** 10/1/15: normal PTH, ? alendronate, 30/11/16: high PTH normal vitD, zol, 10/5/17: high PTH, ? vitD, zol
S5369	10/10/2013	64	F	Included	A6855: Included	5208: Identified as normocalcaemic	Although patient has coeliac disease, she has normal vitD and high urine calcium without supplements - well controlled- no problem in calcium absorption
S5408	19/05/2017	82	F	Excluded - on furosemide	Not identified	Not identified	Identified from hip BMD, 10/2/16: not furosemide, high PTH but low vitD (secondary hyperparathyroidism), **19/5/17: furosemide, high PTH, normal vitD.** 14/6/17: furosemide, high PTH, ?vitD

Table 6-11: Exclusion criteria for patients identified as likely NPHPT.

Thirteen patients out of 28 were excluded. The table also summarises whether these patients were identified in the previous analysis. The bold letters in the comments column, indicate information for the index date. Zol: zoledronic acid

Study number	Persistent	Age	Gender	BMD result	Mean PTH (ng/L)	Mean calcium (mmol/L)	Follow up (years)	Phosphate on index day	ALP on index day	Previous Fractures	Parathyroid	Laboratory investigation
S696	Y	75	F	Osteoporosis, within expected range	74	2.53	6	Normal	Normal	Y		Accelerated bone loss
S1620	Y	75	F	Osteoporosis, below the average	121	2.44	5	Normal	Normal	Y		Low BMD for age
S1753	N	86	M	Osteopenia, within expected range	151	2.58	6	Normal	Normal	N		Workup due to PHPT
S2453	N	57	F	Osteoporosis, below the average	124	2.55	4	Normal	Normal	Y	Patient’s wish: surgery	Low BMD for age
S3812	N	70	F	Osteopenia, within expected range	106	2.62	3	Borderline low (0.8mmol/L)	Normal	N		Follow up
S3882	N	69	F	Osteoporosis, within expected	118	2.51	7	Normal	Normal	N	Surgery: adenoma? hyperplasia?	Vertebral fracture

S4392	N	66	F	Osteoporosis, within expected	185	2.52	6	Normal	High (148 IU/L)	N	FNA: Parathyroid adenoma?	Follow up
S5369	N	64	F	Osteoporosis, below the average	81	2.58	3	Normal	Normal	Y	US: Adenoma? MIBI: Negative	Low BMD for age

Table 6-12: Characteristics of the eight NPHPT patients selected from analysis 3. Y: yes, N: no, F: female; M: male

6.2.3.1.4 Problems identified from Analysis 3

In Analysis 3, patients with likely NPHPT were excluded based on the repeated vitamin D and eGFR measurements. Some were also excluded because they had inconsistent measurements of PTH throughout their follow up. After reviewing this approach, it was decided that a different approach should be followed. According to the definition of NPHPT, PTH should be persistent on several occasions, but not necessarily throughout the follow up. The same is true for vitamin D and eGFR. Checking for follow up results in order to decide about whether a patient should be included in the NPHPT group had been proven troublesome and would not always result in the same decisions. It was also based on speculations like in the case of patient S227, to whom likely chronic vitamin D deficiency was defined as the cause of high PTH. Patients on bisphosphonates on index date and who then stopped, were included if they had only one measurement available consistent with NPHPT after the index date (S696).

Therefore, it was decided to follow a different approach to improve consistency. Inclusion and exclusion criteria on NPHPT would only be based on laboratory results of the index date, information from the medical records (medical problems) and not on follow up results. Once these patients were identified, a further review of them would be possible.

At this point, it should be mentioned that defining NPHPT patients was difficult in this thesis and as will also be mentioned later, I believe that the definition of NPHPT should be updated.

6.2.4 Analysis 4 (completed in 2018)

6.2.4.1 Checking results on index date

Using the data Analysis 3, twenty-eight patients were identified as having likely NPHPT. Going back through their records and only considering the laboratory measurements at the index date, thirteen patients were excluded (Table 6-13).

Study number	**Age**	**Gender**	**Analysis 4: Result of index NPHPT (just from the information from the measurements on index day and the notes)**	**Result in analysis 3**
S189	65	F	Excluded - sodium valproate	Excluded - sodium valproate - only one PTH available
S227	59	F	Included	Excluded - probably chronic vitamin D deficiency
S449	65	F	Excluded - treated pseudohypoparathyroidism	Excluded - treated pseudohypoparathyroidism
S567	72	F	Excluded - tertiary hyperparathyroidism-renal transplant	Excluded - tertiary hyperparathyroidism-renal transplant
S696	75	F	Excluded - on bisphosphonates	Included because one result available after stopping bisphosphonates
S757	68	F	Included	Excluded - secondary hyperparathyroidism (low eGFR)
S871	83	M	Excluded -on bisphosphonates	Excluded - only one PTH-on bisphosphonates
S911	70	F	Included	Excluded - inconsistency of PTH
S1194	74	F	Excluded - on bisphosphonates	Excluded - on bisphosphonates- borderline GFR
S1620	75	F	Included	Included

S1692	79	F	Excluded - on bisphosphonates	Excluded - bisphosphonates
S1753	86	M	Included	Included
S2406	67	F	Included	Excluded - no persistence of high PTH
S2453	57	F	Included	Included
S2654	88	F	Excluded - Crohn's- bisphosphonates- furosemide	Excluded - Crohn's- bisphosphonates- furosemide
S2720	83	F	Included	Excluded - just one measurement of PTH
S3021	64	F	Included	Excluded - inconsistency of PTH
S3703	72	F	Excluded- on carbamazepine	Excluded- on carbamazepine
S3812	70	F	Included	Included
S3820	49	F	Included	Excluded - Inconsistency of PTH
S3841	84	F	Excluded - on bisphosphonates	Excluded - bisphosphonates
S3882	69	F	Included	Included
S4392	66	F	Included	Included
S4618	59	F	Included	Excluded - chronic vitamin D deficiency
S4903	59	M	Excluded - renal transplant	Excluded - renal transplant
S5321	78	F	Excluded - on bisphosphonates	Excluded – bisphosphonates
S5369	64	F	Included	Included
S5408	82	F	Excluded - on furosemide	Excluded - on furosemide

Table 6-13: Checking the 28 likely NPHPT patients for inclusion and exclusion criteria based on the index date.

Thirteen patients were excluded. The inclusions are shown in green

6.2.4.2 Further steps decided after Analysis 4

The next step was to study the natural history of the disease in more detail. Having seen two patterns of variations of calcium (persistent normocalcaemia and intermittent hypercalcaemia), it was decided to also check a group of patients identified with hyperparathyroid hypercalcaemia (HH). By definition, this group mainly consists of PHPT patients, but a small number of FHH patients might be included. As NPHPT patients had normal 25(OH)D and eGFR on their index date, I decided to compare this group with a sample of HH patients having the same characteristics (normal eGFR and vitamin D) on the index date. That could ensure, to some extent, that any variations in calcium were because of the disease and not because of other factors.

The question was whether the patients with primary hyperparathyroidism would have a similar pattern of intermittent hypercalcaemia as the NPHPT patients, and if yes, whether the variation of adjusted calcium was similar between this group and the NPHPT group.

It was also decided that it would be useful to compare these findings with a group of subjects with no problems in calcium metabolism. Based on the analysis with the Mahalanobis distance, 5574 subjects were identified as “normal”. A sample of patients from this population was decided to be used as the control group. I understand that this is a referral centre and all these patients were investigated for secondary osteoporosis, but comparing their results to the groups mentioned above would still give useful information on the variation of adjusted calcium. Once again, the control group should have normal 25(OH)D and eGFR on the index date.

Moreover, as the analysis would involve patients with primary hyperparathyroidism, information on whether these patients had parathyroid surgery would be useful. That

would ensure that all the measurements included in the study of natural history were due to the disease, as all measurements after surgery should be excluded. Having a list of all the parathyroid surgeries performed at Sheffield Teaching Hospitals would help with identifying these patients and supplement the manual check performed through the NHS computers.

6.2.4.3 Problems identified from Analysis 4

Up to this point, all the laboratory measurements (between 2011 and 2017) were extracted manually from ICE by checking each patient's results. ICE contained all the laboratory information performed from 2009 at Sheffield Teaching Hospitals. Once a laboratory evaluation had been reviewed and acted on upon a health care provider, this was then filed. The results that had been filed, were then archived and access to this data was not always possible at the first instance when the patient information was accessed. The way to overcome this was by asking the system to show the archived results and then refresh the page. Once again, this approach had limitations. The page had to be refreshed several times, especially for patients having a lot of information archived. There was no way to know when all the information were seen. There were occasions when going back on ICE on a further occasion, more results not seen on the first time were available. When dealing with a small number of patients, this process could be repeated to ensure that all results were shown. However, as the approach from this point forward was decided to change, it was considered better to ask the IT Department to extract all the information on the patients of interest. This would also extend the follow up period to the time point where the extraction took place and could alter the previous results.

Finally, although the laboratory's manufacturer changed to Roche in 2011, there was also a change in the method used for the measurement of calcium in January 2013 (as described in the Methods in Chapter 2). Although having a long follow up period available (2011-2018) has its advantages, it was considered best to only include measurements after 2013 to ensure that the change in method would not affect the results of the natural history.

6.2.5 Preparation for the analysis 5

As mentioned before, in order to study the natural history of NPHPT in more detail, new databases on the follow up measurements of these patients had to be retrieved. Moreover, the PHPT patients and the control group had to be characterised and databases on available follow up laboratory measurements for these groups also had to be requested.

As the analysis from this point onward would become more challenging and involve a variety of databases and more complex approaches, it was decided to start using R studio for the manipulation of the databases. There were many R codes used throughout these analyses; the ones are included in the Supplementary material.

6.2.5.1 Getting the repeated measurements

Using the final database described above to calculate persistence (Database "SearchServiceResultsSTH15691_AllDXAPatients" (Table 6-7), the study IDs for patients fulfilling the following criteria were retrieved and sent to the IT department.

- For the NPHPT group: patients who on the index date had
    - Normal adjusted calcium
    - High PTH
    - Normal eGFR

  Total number, n=120

  The database *"SearchServiceResults_sheet3NPHPT"* was received in August 2018. It contained information on repeated measurements adjusted

calcium, 25(OH)D, eGFR, PTH, 24h-urine calcium, fractionated urine calcium, calcium and albumin from January 2009 to the end July 2018.

- For the HH group: patients who on the index date had
    - High adjusted calcium
    - High PTH
    - Normal eGFR

    Total number, n=111

    The database *"SearchServiceResults_sheet2HH"* was received in August 2018. It contained information on repeated measurements of adjusted calcium, 25(OH)D, eGFR, PTH, 24h urine calcium, fractionated urine calcium, total calcium and albumin from January 2009 to the end July 2018.

- For the normal population sample: patients who on the index date had
    - Normal eGFR
    - Normal vitamin D

    Total number, n=2292. The first 300 were chosen

    The database *"SearchServiceResults_sheet1Normal"* was received in August 2018. It contained information on repeated measurements of adjusted calcium, 25(OH)D, eGFR, PTH, total calcium and albumin from January 2009 to the end of July 2018.

The codes built would have to look through the files received, confirm the study IDs of the likely NPHPT patients identified before (Table 6-13) and apply the relevant exclusion criteria. After doing so, and in order to be in line with the international guidelines on persistence, only patients with persistently normal adjusted calcium and

elevated PTH on two consecutive occasions would be included in the analysis. The aim was to make graphs of the means and range of values of different analytes in the final NPHPT patients. The same process would be followed for PHPT and the control group.

The codes are included in the Supplementary material. A summary of the process can be seen below.

6.2.5.2 Process of analysing the data and descriptions of R codes used

- Code 2.1. Hospital to Study ID. Once the repeated measurements databases were received, the first thing was to match the Hospital IDs from the new databases to the study IDs used for this project. Code 2.1 went through this process for the NPHPT and HH group. As mentioned above, the NPHPT group consisted of patients who on the index date had normal adjusted calcium, high PTH, normal eGFR (n=120) and the HH group consisted of patients who on the index date had high adjusted calcium, high PTH, normal eGFR (n=111)
- Code 2.2 followed a similar approach for the control group. For the control group, a random sample of 300 subjects was chosen from all the participants being in the ellipse and who on the index date had normal eGFR and normal vitamin D
- Code 2.3. Surgery information. The received database for parathyroidectomies had to be corresponded to the study IDs included in the project. All the patients with NPHPT and HH found and included in the study also had to be looked up on ICE, in case surgery was missed from this list. An updated file containing the subject IDs of the participants of this project was made, and the extra surgery dates found were added manually

- Code 2.4. Confirm the NPHPT patients according to index date. Code 2.4 took the comma-separated values (CSV) files with the repeated biochemical measurements for all the patients described above patients, calculated the adjusted calcium using the equation given in the Methods and formatted the files for further analysis. It kept only the measurements after January 2013 as described above and excluded any measurements after parathyroid surgery. The different statistical values of these measurements were calculated for each patient (maximum, minimum, mean, SD, variance, degrees of freedom).

  The code then selected the patients that fulfilled the biochemical criteria for NPHPT. After doing so, the same 28 likely NPHPT patients were found and the exclusion criteria were applied (Table 6-13). Thirteen patients were excluded. At this point, and in order to be in line with the international guidelines on persistence, only patients with persistently normal adjusted calcium and elevated PTH on two consecutive occasions were included. Therefore, two further patients were excluded because of PTH inconsistency (S2406 and S3820).

  The graphs of the means of the measurements (adjusted calcium, PTH, vitamin D and eGFR) and their ranges in each patient were formed. Finally, the within-subject standard deviation of adjusted calcium was calculated using the approach described in the Methods.

- Code 2.5. A similar process was followed for the HH group in code 2.5. Once again, only patients with consistently high calcium and PTH on at least two consecutive occasions were included. Moreover, in order to only include patients with PHPT in this group, one or more of the following criteria were used:
  - 24-hour urine calcium> 2.5 mmol/24h

- fractional calcium excretion >0.02
- surgically proven PHPT (correction of high calcium after parathyroid surgery).

  This was done to exclude patients with FHH

In more detail, one patient was excluded because of renal transplant done previously (S2706), eight patients due to only one PTH result available (S0444, S5484, S0262, S2842, S6275, S6123, S2187, S4366), one patient due to the fact that they did not fulfil the criterion on persistence of calcium (S0441) and one patient due to the fact that no 24h urine calcium was available to allow the exclusion of FHH (S2031). At this point, all remaining patients in this group were defined as primary hyperparathyroidism (PHPT).

The graphs of the means of the measurements and their ranges in each patient were formed. Finally, the within-subject standard deviation of adjusted calcium was calculated using the approach described in the Methods.

- Code 2.6. The sixth code took the CSV files with the repeated measurements for all the NPHPT and PHPT patients and created their graphs over time
- Code 2.7. This code followed a similar approach with codes 2.4 and 2.5 for the control group
- Code 2.8. This code took the CSV files with information on the selected NPHPT and PHPT patients and the control group and binded the necessary information together to allow further analyses. The graphs of the means of the measurements (PTH and adjusted calcium) and their ranges in each patient

were formed, this time combining information from all groups. Box plots of the information on age, adjusted calcium and PTH of the three groups were formed.

- Code 2.9. This code took the baseline measurements for the three groups (NPHPT and control) and formatted the files for further analysis. The comparisons for baseline characteristics were made (gender distribution, age, different measurements) after checking for the necessary assumptions eg normal distribution of samples. The results were extracted in a table to be used for Chapter 2
- Code 2.10. This code took the files with the repeated measurements for the control group, the NPHPT and PHPT patients and used a mixed linear model as described in the Methods, to compare the overall measurements of PTH and adjusted calcium resulting from follow up.

6.2.6 Final analysis

After the completion of this analysis, a further consideration was given to the definition of hypercalciuria used up to this point (>10 mmol/24h). It was considered best to adapt this to the international definition of 24-hour urine calcium ≥6.25 mmol/24h for women or 7.5 mmol/24h for men and/or ≥ 0.1 mmol/kg/24h (Worcester & Coe, 2008). The results presented in Chapter 3 have implemented this change and two further patients from Analysis 5 have been excluded.

6.2.7 Normocalcaemic hypoparathyroidism study (NHYPO)

There were twenty-two likely NHYPO patients identified in analysis 3. The patients' medical notes were reviewed to look for other causes of the abnormalities (non-PTH induced hypocalcaemia due to cancer) and some patients were excluded. Any unconfirmed data were also excluded. The remaining patients were used for the analysis described in chapter 3 (final one). Their repeated calcium, albumin and PTH measurements were retrieved manually for the hospital laboratory software (ICE). The analysis was done in R studio and final codes used can be found in the Supplementary material. The process followed in the different R codes is summarised below.

- Code 3.1. The first code took the comma-separated values (CSV) files with the repeated calcium, albumin and PTH measurements for all the NHYPO patients, calculated the adjusted calcium using the equation given in the Methods and formatted the files for further analysis. It kept only the measurements after January 2013 as described above. The different statistical values of these measurements were calculated for each patient (maximum, minimum, mean, SD, variance, degrees of freedom). The graphs of the means of the

measurements (PTH and adjusted calcium) and their ranges in each patient were formed. Finally, the within-subject standard deviation was calculated using the approach described in the Methods

- Code 3.2. The second code took the CSV files with the repeated adjusted calcium and PTH measurements for all the NHYPO patients and created their graphs over time
- Code 3.3. The third code took the CSV files with information on the selected NHYPO patients and the control group and binded the necessary information together to allow further analyses. For the control group, a random sample of 300 subjects was chosen from all the participants being in the ellipse and who on the index date had normal eGFR and normal vitamin D. The graphs of the means of the measurements (PTH and adjusted calcium) and their ranges in each patient were formed, this time combining information from both groups. Box plots of the information on age, adjusted calcium and PTH of the two groups were formed and basic statistical comparisons were made
- Code 3.4. The fourth code used the baseline measurements for the two groups (NHYPO and control) and formatted the files for further analysis. The comparisons for baseline characteristics were made (gender distribution, age, different measurements) after checking for the necessary assumptions eg normal distribution of samples. The results were extracted in a table to be used for Chapter 3
- Code 3.5. The fifth code used the files with the repeated measurements for the control group and the NHYPO group and used a mixed linear model as described in the Methods, to compare the overall measurements of PTH and adjusted calcium resulting from follow up

- Code 3.6. Finally, the sixth code compared the persistent versus non-persistent NHYPO patients and looked for any statistical differences in age, gender and biochemical measurements

Section 3: Bibliography

Abate, E. G. & Clarke, B. L. (2016) Review of Hypoparathyroidism. *Front Endocrinol (Lausanne)*, 7, 172.

Altman, D. (1991) *Practical statistics for medical research*. United States of America: Chapman & Hall/ CRC.

Alyne layane pereira, L., Sergio ricardo de lima, A., Lívia laeny henrique, P., Patricia moura cravo, T., Elba, B., Leonardo c, B. & Francisco, B. (2019) High Rate of Occult Urolithiasis in Normocalcemic Primary Hyperparathyroidism. *Kidney & Blood Pressure Research*, 44(5), 1189-1195.

Amaral, L. M., Queiroz, D. C., Marques, T. F., Mendes, M. & Bandeira, F. (2012) Normocalcemic versus Hypercalcemic Primary Hyperparathyroidism: More Stone than Bone? *J Osteoporos*, 2012, 128352.

Aspray, T. J., Bowring, C., Fraser, W., Gittoes, N., Javaid, M. K., Macdonald, H., Patel, S., Selby, P., Tanna, N. & Francis, R. M. (2014) National Osteoporosis Society Vitamin D Guideline Summary. *Age and Ageing*, 43(5), 592-595.

Astor, M. C., Lovas, K., Debowska, A., Eriksen, E. F., Evang, J. A., Fossum, C., Fougner, K. J., Holte, S. E., Lima, K., Moe, R. B., Myhre, A. G., Kemp, E. H., Nedrebo, B. G., Svartberg, J. & Husebye, E. S. (2016) Epidemiology and Health-Related Quality of Life in Hypoparathyroidism in Norway. *J Clin Endocrinol Metab*, 101(8), 3045-53.

Ayturk, S., Gursoy, A., Bascil Tutuncu, N., Ertugrul, D. T. & Guvener Demirag, N. (2006) Changes in insulin sensitivity and glucose and bone metabolism over time in patients with asymptomatic primary hyperparathyroidism. *J Clin Endocrinol Metab*, 91(11), 4260-3.

Baird, G. S. (2011) Ionized calcium. *Clin Chim Acta*, 412(9-10), 696-701.

Bandeira, L. & Bilezikian, J. (2016) Primary Hyperparathyroidism. *F1000Res*, 5.

Barth, J. H., Fiddy, J. B. & Payne, R. B. (1996) Adjustment of Serum Total Calcium for Albumin Concentration: Effects of Non-Linearity and of Regression Differences between Laboratories. *Annals of Clinical Biochemistry*, 33(1), 55-58.

BeckmanCoulter (2012) *Calcium (Arsenazo)*, 2012. Available online: https://www.beckmancoulter.com/wsrportal/techdocs?docname=/cis/BAOSR6x117/%%/EN_CALCIUM [Accessed.

BeckmanCoulter (2013) *Albumin*, 2013. Available online: https://www.beckmancoulter.com/wsrportal/techdocs?docname=/cis/BAOSR6X02/%25%25/EN_ALBUMIN_BAOSR6x02_US.pdf [Accessed.

BeckmanCoulter (2015) *Creatinine enzymatic*, 2015. Available online: https://www.beckmancoulter.com/wsrportal/techdocs?docname=/cis/A69463/%%/EN_CR-E.pdf [Accessed.

Berg, J. (2012) The Approach to Pathology Harmony in the UK. *Clin Biochem Rev*, 33(3), 89-93.

Berg, J. (2014) The UK Pathology Harmony initiative; The foundation of a global model. *Clinica Chimica Acta*, 432, 22-26.

Berger, C., Almohareb, O., Langsetmo, L., Hanley, D. A., Kovacs, C. S., Josse, R. G., Adachi, J. D., Prior, J. C., Towheed, T., Davison, K. S., Kaiser, S. M., Brown, J. P. & Goltzman, D. (2015) Characteristics of hyperparathyroid states in the Canadian multicentre osteoporosis study (CaMos) and relationship to skeletal markers. *Clinical Endocrinology*, 82(3), 359-368.

Bertocchio, H. J.-P., Tafflet, H. M., Koumakis, H. E., Maruani, H. G., Vargas-Poussou, H. R., Silve, H. C., Nissen, H. P., Baron, H. S., Prot-Bertoye, H. C., Courbebaisse, H.

M., Souberbielle, H. J.-C., Rejnmark, H. L., Cormier, H. C. & Houillier, H. P. (2018) Pro- FHH: A Risk Equation to Facilitate the Diagnosis of Parathyroid- Related Hypercalcemia. *The Journal of Clinical Endocrinology & Metabolism*, 103(7), 2534-2542.

Beysel, S., Caliskan, M., Kizilgul, M., Apaydin, M., Kan, S. & Ozbek, M. (2019) Parathyroidectomy improves cardiovascular risk factors in normocalcemic and hypercalcemic primary hyperparathyroidism. *BMC Cardiovascular Disorders*, 19(1).

Bikle, D. D., Adams, J. S. & Christakos, S. (2018) Vitamin D. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, 230-240.

Bilezikian, J. P., Brandi, M. L., Eastell, R., Silverberg, S. J., Udelsman, R., Marcocci, C. & Potts, J. T., Jr. (2014) Guidelines for the management of asymptomatic primary hyperparathyroidism: summary statement from the Fourth International Workshop. *J Clin Endocrinol Metab*, 99(10), 3561-9.

Biondi, B., Bartalena, L., Cooper, D. S., Hegedüs, L., Laurberg, P. & Kahaly, G. J. (2015) The 2015 European Thyroid Association Guidelines on Diagnosis and Treatment of Endogenous Subclinical Hyperthyroidism. *European Thyroid Journal*, 4(3), 149-163.

Blake, G., E. Adams, J. & Bishop, N. (2013) DXA in Adults and Children, *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*John Wiley & Sons, Inc., 249-263.

Bland, J. M. & Altman, D. G. (1994) Statistics Notes: Some examples of regression towards the mean. *BMJ*, 309(6957), 780.

Bland, J. M. & Altman, D. G. (1996a) Statistics Notes: Measurement error. *BMJ*, 313(7059), 744.

Bland, J. M. & Altman, D. G. (1996b) Statistics Notes: Measurement error proportional to the mean (British Medical Journal (1996) July (106)). *British Medical Journal*, 313(7059), 744.

Bland, M. (2011) What is the standard error of the within-subject

standard deviation, sw?

Bland, M. (2015) *An Introduction to Medical Statistics*, Fourth edition. Oxford University Press.

Boink, A. B., Buckley, B. M., Christiansen, T. F., Covington, A. K., Maas, A. H., Muller-Plathe, O., Sachs, C. & Siggaard-Andersen, O. (1991) IFCC recommendation on sampling, transport and storage for the determination of the concentration of ionized calcium in whole blood, plasma and serum. *J Automat Chem*, 13(5), 235-9.

Bollerslev, J., Rejnmark, L., Marcocci, C., Shoback, D. M., Sitges-Serra, A., van Biesen, W. & Dekkers, O. M. (2015) European Society of Endocrinology Clinical Guideline: Treatment of chronic hypoparathyroidism in adults. *Eur J Endocrinol*, 173(2), G1-20.

Bollerslev, J., Schalin-Jäntti, C., Rejnmark, L., Siggelkow, H., Morreau, H., Thakker, R., Sitges-Serra, A., Cetani, F. & Marcocci, C. (2019) Unmet therapeutic, educational and scientific needs in parathyroid disorders: Consensus Statement from the first European Society of Endocrinology Workshop (PARAT). *European Journal of Endocrinology*, 181(3), P1.

Bossert, I., Chytiris, S., Hodolic, M., Croce, L., Mansi, L., Chiovato, L., Mariani, G. & Trifirò, G. (2019) PETC/ CT with 18F- Choline localizes hyperfunctioning parathyroid adenomas equally well in normocalcemic hyperparathyroidism as in overt hyperparathyroidism. *Journal of endocrinological investigation*, 42(4), 419-426.

Brandi, M. L., Bilezikian, J. P., Shoback, D., Bouillon, R., Clarke, B. L., Thakker, R. V., Khan, A. A. & Potts, J. T., Jr. (2016) Management of Hypoparathyroidism: Summary Statement and Guidelines. *J Clin Endocrinol Metab*, 101(6), 2273-83.

Brardi, S., Cevenini, G., Verdacchi, T., Romano, G. & Ponchietti, R. (2015) Use of cinacalcet in nephrolithiasis associated with normocalcemic or hypercalcemic primary hyperparathyroidism: results of a prospective randomized pilot study. *Arch Ital Urol Androl*, 87(1), 66-71.

Cakir, I., Unluhizarci, K., Tanriverdi, F., Elbuken, G., Karaca, Z. & Kelestimur, F. (2012) Investigation of insulin resistance in patients with normocalcemic primary hyperparathyroidism. *Endocrine*, 42(2), 419-22.

Calvo, M. S., Eastell, R., Offord, K. P., Bergstralh, E. J. & Burritt, M. F. (1991) Circadian variation in ionized calcium and intact parathyroid hormone: evidence for sex differences in calcium homeostasis. *J Clin Endocrinol Metab*, 72(1), 69-76.

Carrivick, J. S., Walsh, P. J., Brown, J. S., Wardrop, C. R. & Hadlow, C. N. (2015) Brief Report: Does PTH Increase With Age, Independent of 25- Hydroxyvitamin D, Phosphate, Renal Function, and Ionized Calcium? *The Journal of Clinical Endocrinology & Metabolism*, 100(5), 2131-2134.

Castrillon, J. L., Diaz-Soto, G., Jauregui, O. I., Romero, E. & de Luis Roman, D. (2015) Polymorphisms of the vitamin D receptor and their effect on bone mass density in patients with normocalcemic hyperparathyroidism. *Endocrine*, 50(3), 816-8.

Cesareo, R., Di Stasio, E., Vescini, F., Campagna, G., Cianni, R., Pasqualini, V., Romitelli, F., Grimaldi, F., Manfrini, S. & Palermo, A. (2015) Effects of alendronate and vitamin D in patients with normocalcemic primary hyperparathyroidism. *Osteoporos Int*, 26(4), 1295-302.

Chen, G., Xue, Y., Zhang, Q., Xue, T., Yao, J., Huang, H., Liang, J., Li, L., Lin, W., Lin, L., Shi, L., Cai, L. & Wen, J. (2015) Is Normocalcemic Primary Hyperparathyroidism Harmful or Harmless? *J Clin Endocrinol Metab*, 100(6), 2420-4.

Christensen, S. E., Nissen, P. H., Vestergaard, P., Heickendorff, L., Brixen, K. & Mosekilde, L. (2008) Discriminative power of three indices of renal calcium excretion for the distinction between familial hypocalciuric hypercalcaemia and primary hyperparathyroidism: a follow-up study on methods. *Clin Endocrinol (Oxf)*, 69(5), 713-20.

Clapham, M. (2016) Mahalanobis distance.

Clarke, B. L., Brown, E. M., Collins, M. T., Juppner, H., Lakatos, P., Levine, M. A., Mannstadt, M. M., Bilezikian, J. P., Romanischen, A. F. & Thakker, R. V. (2016) Epidemiology and Diagnosis of Hypoparathyroidism. *J Clin Endocrinol Metab*, 101(6), 2284-99.

Cohn, D. V. & MacGregor, R. R. (1981) The biosynthesis, intracellular processing, and secretion of parathormone. *Endocr Rev*, 2(1), 1-26.

Cooper, M. S. & Gittoes, N. J. L. (2008) Diagnosis and management of hypocalcaemia. *BMJ*, 336(7656), 1298.

Crowley, R. K. & Gittoes, N. J. (2016) Elevated PTH with normal serum calcium level: a structured approach. *Clinical endocrinology*, 84(6), 809-813.

Cunha-Bezerra, P., Vieira, R., Amaral, F., Cartaxo, H., Lima, T., Montarroyos, U. & Bandeira, F. (2018) Better performance of four- dimension computed tomography as a localization procedure in normocalcemic primary hyperparathyroidism. *Journal of Medical Imaging and Radiation Oncology*, 62(4), 493-498.

Cusano, N., Rubin, M., Sliney, J. & Bilezikian, J. (2012) Mini- review: new therapeutic options in hypoparathyroidism. *International Journal of Basic and Clinical Endocrinology*, 41(3), 410-414.

Cusano, N. E., Maalouf, N. M., Wang, P. Y., Zhang, C., Cremers, S. C., Haney, E. M., Bauer, D. C., Orwoll, E. S. & Bilezikian, J. P. (2013a) Normocalcemic hyperparathyroidism and hypoparathyroidism in two community-based nonreferral populations. *J Clin Endocrinol Metab*, 98(7), 2734-41.

Cusano, N. E., Silverberg, S. J. & Bilezikian, J. P. (2013b) Normocalcemic primary hyperparathyroidism. *J Clin Densitom*, 16(1), 33-9.

Dent, D. M., Miller, J. L., Klaff, L. & Barron, J. (1987) The incidence and causes of hypercalcaemia. *Postgraduate Medical Journal*, 63(743), 745.

Diasorin (2013) Diasorin Liaison 25-OH vitamin D total assay U.S. Food and Drug Administration approval summary. Available online: https://www.accessdata.fda.gov/cdrh_docs/pdf13/K132492.pdf [Accessed.

Diaz-Soto, G., Romero, E., Castrillon, J. L., Jauregui, O. I. & de Luis Roman, D. (2015) Clinical Expression of Calcium Sensing Receptor Polymorphism (A986S) in Normocalcemic and Asymptomatic Hyperparathyroidism. *Horm Metab Res*.

Diri, H., Unluhizarci, K. & Kelestimur, F. (2014) Investigation of glucose intolerance in patients with normocalcemic primary hyperparathyroidism: 4-year follow-up. *Endocrine*, 47(3), 971-2.

Dorairajan, N. & Pradeep, P. V. (2014) Vignette hyperparathyroidism: glimpse into its history. *Int Surg*, 99(5), 528-33.

Díaz-Soto, G., Romero, E., Castrillón, J. L. P., Jauregui, O. I. & de Luis Román, D. (2016) Clinical Expression of Calcium Sensing Receptor Polymorphism (A986S) in

Normocalcemic and Asymptomatic Hyperparathyroidism. *Horm Metab Res*, 48(03), 163-168.

Eastell, R., Arnold, A., Brandi, M. L., Brown, E. M., D'Amour, P., Hanley, D. A., Rao, D. S., Rubin, M. R., Goltzman, D., Silverberg, S. J., Marx, S. J., Peacock, M., Mosekilde, L., Bouillon, R. & Lewiecki, E. M. (2009) Diagnosis of asymptomatic primary hyperparathyroidism: proceedings of the third international workshop. *J Clin Endocrinol Metab*, 94(2), 340-50.

Eastell, R., Brandi, M. L., Costa, A. G., D'Amour, P., Shoback, D. M. & Thakker, R. V. (2014) Diagnosis of asymptomatic primary hyperparathyroidism: proceedings of the Fourth International Workshop. *J Clin Endocrinol Metab*, 99(10), 3570-9.

Eastell, R., Calvo, M. S., Burritt, M. F., Offord, K. P., Russell, R. G. & Riggs, B. L. (1992) Abnormalities in circadian patterns of bone resorption and renal calcium conservation in type I osteoporosis. *J Clin Endocrinol Metab*, 74(3), 487-94.

Eknoyan, G. (1995) A history of the parathyroid glands. *Am J Kidney Dis*, 26(5), 801-7.

el-Hajj Fuleihan, G., Klerman, E. B., Brown, E. N., Choe, Y., Brown, E. M. & Czeisler, C. A. (1997) The parathyroid hormone circadian rhythm is truly endogenous--a general clinical research center study. *J Clin Endocrinol Metab*, 82(1), 281-6.

Favus, M. J. & Goltzman, D. (2013) Regulation of Calcium and Magnesium, *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*John Wiley & Sons, Inc., 171-179.

Ferré-Masferrer, M., Fuentes-Arderiu, X. & Puchal-Añé, R. (1999) Indirect reference limits estimated from patients' results by three mathematical procedures. *Clinica Chimica Acta*, 279(1-2), 97-105.

Fraser, C. (2013) *Biologic variation: From principles to practice*, Seventh printing, 2013 edition. Amer Assn for Clinical Chemistry.

Fraser, W. D., Logue, F. C., Christie, J. P., Gallacher, S. J., Cameron, D., O'Reilly, D. S., Beastall, G. H. & Boyle, I. T. (1998) Alteration of the circadian rhythm of intact parathyroid hormone and serum phosphate in women with established postmenopausal osteoporosis. *Osteoporos Int*, 8(2), 121-6.

Garcia-Martin, A., Reyes-Garcia, R. & Munoz-Torres, M. (2012) Normocalcemic primary hyperparathyroidism: one-year follow-up in one hundred postmenopausal women. *Endocrine*, 42(3), 764-6.

Gardella, T., Juppner, H., Brown, E., Kronenberg, H. & Potts, J. J. (2010) Parathyroid Hormone and Parathyroid Hormone-Related Peptide in the Regulation of Calcium Homeostasis and Bone Development, in De Grout, L. & Jameson, L. (eds), *Endocrinology*, 6th editionSaunders Elsevier.

Gardella, T. J. & Vilardaga, J. P. (2015) International Union of Basic and Clinical Pharmacology. XCIII. The parathyroid hormone receptors--family B G protein-coupled receptors. *Pharmacol Rev*, 67(2), 310-37.

Giusti, A., Barone, A., Pioli, G., Girasole, G., Razzano, M., Pizzonia, M., Pedrazzoni, M., Palummeri, E. & Bianchi, G. (2010) Heterogeneity in serum 25-hydroxy-vitamin D response to colecalciferol in elderly women with secondary hyperparathyroidism and vitamin D deficiency. *J Am Geriatr Soc*, 58(8), 1489-95.

Goldstein, D. (1990) *Serum Calcium (Chapter 143) in Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition* Boston: Butterworths.

Gómez-Ramírez, J., Gómez-Valdazo, A., Luengo, P., Porrero, B., Osorio, I. & Rivas, S. (2019) Comparative prospective study on the presentation of normocalcemic

primary hyperparathyroidism. Is it more aggressive than the hypercalcemic form? *American journal of surgery*.

Hagstrom, E., Lundgren, E., Rastad, J. & Hellman, P. (2006) Metabolic abnormalities in patients with normocalcemic hyperparathyroidism detected at a population-based screening. *Eur J Endocrinol*, 155(1), 33-9.

Hannan, F. M. & Thakker, R. V. (2013) Calcium-sensing receptor (CaSR) mutations and disorders of calcium, electrolyte and water metabolism. *Best Pract Res Clin Endocrinol Metab*, 27(3), 359-71.

Hendy, G. N. (2018) Calcium-Sensing Receptor. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, 221-229.

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., Murad, M. H. & Weaver, C. M. (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*, 96(7), 1911-30.

Holick, M. F., Siris, E. S., Binkley, N., Beard, M. K., Khan, A., Katzer, J. T., Petruschke, R. A., Chen, E. & de Papp, A. E. (2005) Prevalence of Vitamin D Inadequacy among Postmenopausal North American Women Receiving Osteoporosis Therapy. *The Journal of Clinical Endocrinology & Metabolism*, 90(6), 3215-3224.

https://www.iscd.org.

Huang, Y., Zhou, Y., Yang, W., Butters, R., Lee, H. W., Li, S., Castiblanco, A., Brown, E. M. & Yang, J. J. (2007) Identification and dissection of Ca(2+)-binding sites in the extracellular domain of Ca(2+)-sensing receptor. *J Biol Chem*, 282(26), 19000-10.

ISCD (2019) Adult Official Positions of the International Society for Clinical Densitometry (ISCD).

Johansson, H. (2015) The Uppsala anatomist Ivar Sandstrom and the parathyroid gland. *Ups J Med Sci*, 120(2), 72-7.

Kafetzis, I. D., Diamantopoulos, A., Christakis, I. & Leoutsakos, B. (2011) The history of the parathyroid glands. *Hormones (Athens)*, 10(1), 80-4.

Kanis, J. A., McCloskey, E. V., Johansson, H., Oden, A., Melton, L. J. & Khaltaev, N. (2008) A reference standard for the description of osteoporosis. *Bone*, 42(3), 467-475.

KDIGO (2012) KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease.

Khan, A. A., Hanley, D. A., Rizzoli, R., Bollerslev, J., Young, J. E. M., Rejnmark, L., Thakker, R., D Amour, P., Paul, T., van Uum, S., Shrayyef, M. Z., Goltzman, D., Kaiser, S., Cusano, N. E., Bouillon, R., Mosekilde, L., Kung, A. W., Rao, S. D., Bhadada, S. K., Clarke, B. L., Liu, J., Duh, Q., Lewiecki, E. M., Bandeira, F., Eastell, R., Marcocci, C., Silverberg, S. J., Udelsman, R., Davison, K. S., Potts, J. T., Brandi, M. L. & Bilezikian, J. P. (2017) Primary hyperparathyroidism: review and recommendations on evaluation, diagnosis, and management. A Canadian and international consensus.

Kiriakopoulos, A., Petralias, A. & Linos, D. (2018) Classic Primary Hyperparathyroidism Versus Normocalcemic and Normohormonal Variants: Do They Really Differ? *Official Journal of the International Society of Surgery/Société Internationale de Chirurgie*, 42(4), 992-997.

Kontogeorgos, G., Trimpou, P., Laine, C. M., Olerod, G., Lindahl, A. & Landin-Wilhelmsen, K. (2015) Normocalcaemic, vitamin D-sufficient hyperparathyroidism - high prevalence and low morbidity in the general population: A long-term follow-up study, the WHO MONICA project, Gothenburg, Sweden. *Clin Endocrinol (Oxf)*, 83(2), 277-84.

Koumakis, E., Souberbielle, J. C., Payet, J., Sarfati, E., Borderie, D., Kahan, A. & Cormier, C. (2014) Individual site-specific bone mineral density gain in normocalcemic primary hyperparathyroidism. *Osteoporos Int*, 25(7), 1963-8.

Koumakis, E., Souberbielle, J. C., Sarfati, E., Meunier, M., Maury, E., Gallimard, E., Borderie, D., Kahan, A. & Cormier, C. (2013) Bone mineral density evolution after successful parathyroidectomy in patients with normocalcemic primary hyperparathyroidism. *J Clin Endocrinol Metab*, 98(8), 3213-20.

Labrtorian (2017) *Mining Your Routine Data for Reference Intervals: Hoffmann, Bhattacharya and Maximum Likelihood*, 2017. Available online: https://labrtorian.com/tag/bhattacharya/ [Accessed.

Levey, A. S., Bosch, J. P., Lewis, J. B., Greene, T., Rogers, N. & Roth, D. (1999) A more accurate method to estimate glomerular filtration rate from serum creatinine: A new prediction equation. *Annals of Internal Medicine*, 130(6), 461-470.

Levey, A. S., Coresh, J., Greene, T., Stevens, L. A., Zhang, Y. L., Hendriksen, S., Kusek, J. W. & Van Lente, F. (2006) Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Annals of internal medicine*, 145(4), 247-254.

Levey, A. S., Stevens, L. A., Schmid, C. H., Zhang, Y. L., Castro, A. F., Feldman, H. I., Kusek, J. W., Eggers, P., Van Lente, F., Greene, T. & Coresh, J. (2009) A new equation to estimate glomerular filtration rate. *Annals of internal medicine*, 150(9), 604-612.

Lian, I. A. & Åsberg, A. (2018) Should total calcium be adjusted for albumin? A retrospective observational study of laboratory data from central Norway. *BMJ open*, 8(4), e017703-e017703.

Lim, J. Y., Herman, M. C., Bubis, L., Epelboym, I., Allendorf, J. D., Chabot, J. A., Lee, J. A. & Kuo, J. H. (2017) Differences in single gland and multigland disease are seen in low biochemical profile primary hyperparathyroidism. *Surgery*, 161(1), 70-77.

Liu, M., Sum, M., Cong, E., Colon, I., Bucovsky, M., Williams, J., Kepley, A., Kuo, J., Lee, J. A., Lazar, R. M., Marshall, R., Silverberg, S. & Walker, M. D. (2019) Cognition and cerebrovascular function in primary hyperparathyroidism before and after parathyroidectomy. *Journal of endocrinological investigation*.

Lowe, H., McMahon, D. J., Rubin, M. R., Bilezikian, J. P. & Silverberg, S. J. (2007) Normocalcemic Primary Hyperparathyroidism: Further Characterization of a New Clinical Phenotype. *The Journal of Clinical Endocrinology & Metabolism*, 92(8), 3001-3005.

Lundgren, E., Rastad, J., Thrufjell, E., Akerstrom, G. & Ljunghall, S. (1997) Population-based screening for primary hyperparathyroidism with serum calcium and parathyroid hormone values in menopausal women. *Surgery*, 121(3), 287-94.

Magno, A. L., Ward, B. K. & Ratajczak, T. (2011) The calcium-sensing receptor: a molecular perspective. *Endocr Rev*, 32(1), 3-30.

Malabanan, A., Veronikis, I. & Holick, M. (1998) Redefining vitamin D insufficiency. *The Lancet*, 351(9105), 805-806.

Markowitz, M., Rotkin, L. & Rosen, J. F. (1981) Circadian rhythms of blood minerals in humans. *Science*, 213(4508), 672-4.

Markowitz, M. E., Arnaud, S., Rosen, J. F., Thorpy, M. & Laximinarayan, S. (1988) Temporal interrelationships between the circadian rhythms of serum parathyroid hormone and calcium concentrations. *J Clin Endocrinol Metab*, 67(5), 1068-73.

Marques, T. F., Vasconcelos, R., Diniz, E., Rego, D., Griz, L. & Bandeira, F. (2011) Normocalcemic primary hyperparathyroidism in clinical practice: an indolent condition or a silent threat? *Arq Bras Endocrinol Metabol*, 55(5), 314-7.

Maruani, G., Hertig, A., Paillard, M. & Houillier, P. (2003) Normocalcemic primary hyperparathyroidism: evidence for a generalized target-tissue resistance to parathyroid hormone. *J Clin Endocrinol Metab*, 88(10), 4641-8.

Masse, J. (1997) Measurement error. A constant within subject standard deviation cannot be assumed a priori. *British Medical Journal*, 314(7074), 147.

Mesquita, P., Dornelas Leão Leite, A. P., Chagas Crisostomo, S., Veras Filho, E., Cunha Xavier, L. & Bandeira, F. (2017) Evaluation of coronary calcium score in patients with normocalcemic primary hyperparathyroidism. *Vascular Health and Risk Management*, 13, 225-229.

Mitchell, D. M., Regan, S., Cooley, M. R., Lauter, K. B., Vrla, M. C., Becker, C. B., Burnett-Bowie, S. A. & Mannstadt, M. (2012) Long-term follow-up of patients with hypoparathyroidism. *J Clin Endocrinol Metab*, 97(12), 4507-14.

Murray, T. M., Rao, L. G., Divieti, P. & Bringhurst, F. R. (2005) Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl- terminal ligands. *Endocr Rev*, 26(1), 78-113.

Need, G. A., O'loughlin, D. P., Morris, A. H., Horowitz, E. C. M. & Nordin, E. C. B. (2004) The Effects of Age and Other Variables on Serum Parathyroid Hormone in Postmenopausal Women Attending an Osteoporosis Center. *The Journal of Clinical Endocrinology & Metabolism*, 89(4), 1646-1649.

Nesbit, M. A., Hannan, F. M., Howles, S. A., Babinsky, V. N., Head, R. A., Cranston, T., Rust, N., Hobbs, M. R., Heath, H., 3rd & Thakker, R. V. (2013a) Mutations affecting

G-protein subunit alpha11 in hypercalcemia and hypocalcemia. *N Engl J Med*, 368(26), 2476-86.

Nesbit, M. A., Hannan, F. M., Howles, S. A., Reed, A. A., Cranston, T., Thakker, C. E., Gregory, L., Rimmer, A. J., Rust, N., Graham, U., Morrison, P. J., Hunter, S. J., Whyte, M. P., McVean, G., Buck, D. & Thakker, R. V. (2013b) Mutations in AP2S1 cause familial hypocalciuric hypercalcemia type 3. *Nat Genet*, 45(1), 93-7.

NICE (2018) *Vitamin D deficiency in adults-treatment and prevention*, 2018. Available online: https://cks.nice.org.uk/vitamin-d-deficiency-in-adults-treatment-and-prevention#!scenario [Accessed.

NICE (2019) *Hyperparathyroidism (primary): diagnosis, assessment and initial management* National Institute for Health and Care Excellence (UK).

Norman, J., Goodman A Fau - Politz, D. & Politz, D. (2011) Calcium, parathyroid hormone, and vitamin D in patients with primary hyperparathyroidism: normograms developed from 10,000 cases. *Endocr Pract*, May-Jun;17(3), 384-94.

Ott, S., LaCroix, A., Scholes, D., Ichikawa, L. & Wu, K. (2008) Effects of three years of low- dose thiazides on mineral metabolism in healthy elderly persons. *With other metabolic bone diseases*, 19(9), 1315-1322.

Pack, A. M. (2003) The Association between Antiepileptic Drugs and Bone Disease. Los Angeles, CA.

Palermo, A., Jacques, R., Gossiel, F., Reid, D. M., Roux, C., Felsenberg, D., Glueer, C.-C. & Eastell, R. (2015) Normocalcaemic hypoparathyroidism: prevalence and effect on bone status in older women. The OPUS study. *Clinical endocrinology*, 82(6), 816-23.

Pandian, T. K., Lubitz, C. C., Bird, S. H., Kuo, L. E. & Stephen, A. E. (2019) Normocalcemic hyperparathyroidism: A Collaborative Endocrine Surgery Quality Improvement Program analysis. *Surgery*.

Pawlowska, M. & Cusano, N. E. (2015) An overview of normocalcemic primary hyperparathyroidism. *Curr Opin Endocrinol Diabetes Obes*, 22(6), 413-21.

Pedersen, K. O. (1972) On the cause and degree of intraindividual serum calcium variability. *Scand J Clin Lab Invest*, 30(2), 191-9.

Pierreux, J., Bravenboer, B., Velkeniers, B., Unuane, D., Andreescu, C. E. & Vanhoeij, M. (2018) Normocalcemic Primary Hyperparathyroidism: A Comparison with the Hypercalcemic Form in a Tertiary Referral Population. *Horm Metab Res*, 50(11), 797-802.

Pludowski, P., Holick, M. F., Grant, W. B., Konstantynowicz, J., Mascarenhas, M. R., Haq, A., Povoroznyuk, V., Balatska, N., Barbosa, A. P., Karonova, T., Rudenka, E., Misiorowski, W., Zakharova, I., Rudenka, A., Łukaszkiewicz, J., Marcinowska-Suchowierska, E., Łaszcz, N., Abramowicz, P., Bhattoa, H. P. & Wimalawansa, S. J. (2018) Vitamin D supplementation guidelines. *Journal of Steroid Biochemistry and Molecular Biology*, 175, 125-135.

Rapuri, P. B., Kinyamu, H. K., Gallagher, J. C. & Haynatzka, V. (2002) Seasonal changes in calciotropic hormones, bone markers, and bone mineral density in elderly women. *J Clin Endocrinol Metab*, 87(5), 2024-32.

Rejnmark, L., Amstrup, A. K., Mollerup, C. L., Heickendorff, L. & Mosekilde, L. (2013) Further insights into the pathogenesis of primary hyperparathyroidism: a nested case-control study. *J Clin Endocrinol Metab*, 98(1), 87-96.

Rejnmark, L., Vestergaard, P., Heickendorff, L., Andreasen, F. & Mosekilde, L. (2001) Effects of thiazide- and loop-diuretics, alone or in combination, on calcitropic

hormones and biochemical bone markers: a randomized controlled study. *J Intern Med*, 250(2), 144-53.

Rejnmark, L., Vestergaard, P., Heickendorff, L., Andreasen, F. & Mosekilde, L. (2005) Effects of long- term treatment with loop diuretics on bone mineral density, calcitropic hormones and bone turnover. *Journal of Internal Medicine*, 257(2), 176-184.

Rejnmark, L., Vestergaard, P., Heickendorff, L. & Mosekilde, L. (2011) Determinants of plasma PTH and their implication for defining a reference interval. *Clinical Endocrinology*, 74(1), 37-43.

Roche (2019), 2019. Available online: https://dialog1.roche.com/gb/en_gb/eLabDoc [Accessed.

Ross, A. C., Manson, J. E., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., Durazo-Arvizu, R. A., Gallagher, J. C., Gallo, R. L., Jones, G., Kovacs, C. S., Mayne, S. T., Rosen, C. J. & Shapses, S. A. (2011) The 2011 Report on Dietary Reference Intakes for Calcium and Vitamin D from the Institute of Medicine: What Clinicians Need to Know. *The Journal of Clinical Endocrinology & Metabolism*, 96(1), 53-58.

Rosário, P. W. & Calsolari, M. R. (2019) Normocalcemic Primary Hyperparathyroidism in Adults Without a History of Nephrolithiasis or Fractures: A Prospective Study. *Horm Metab Res*, 51(04), 243-247.

Rousseeuw, P. J. & van Zomeren, B. C. (1990) Unmasking Multivariate Outliers and Leverage Points. *Journal of the American Statistical Association*, 85(411), 633-639.

Rubin, M. R., Bilezikian, J. P., McMahon, D. J., Jacobs, T., Shane, E., Siris, E., Udesky, J. & Silverberg, S. J. (2008) The Natural History of Primary Hyperparathyroidism with or without Parathyroid Surgery after 15 Years. *The Journal of Clinical Endocrinology & Metabolism*, 93(9), 3462-3470.

Rubin, M. R. & Levine, M. A. (2013) Hypoparathyroidism and Pseudohypoparathyroidism, *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*John Wiley & Sons, Inc., 579-589.

Shibli-Rahhal, A. & Paturi, B. (2014) Variations in parathyroid hormone concentration in patients with low 25 hydroxyvitamin D. *Osteoporos Int*, 25(7), 1931-6.

Sho, S., Kuo, E., Chen, A., Li, N., Yeh, M. & Livhits, M. (2019) Biochemical and Skeletal Outcomes of Parathyroidectomy for Normocalcemic (Incipient) Primary Hyperparathyroidism. *Annals of Surgical Oncology*, 26(2), 539-546.

Silva, B. C. & Bilezikian, J. P. (2015) Parathyroid hormone: anabolic and catabolic actions on the skeleton. *Current Opinion in Pharmacology*, 22, 41-50.

Silva, B. C., Cusano, N. E. & Bilezikian, J. P. (2018) Primary hyperparathyroidism. *Best Practice & Research Clinical Endocrinology & Metabolism*, 32(5), 593-607.

Silverberg, S. J. (2013) Primary Hyperparathyroidism, *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*John Wiley & Sons, Inc., 543-552.

Silverberg, S. J. & Bilezikian, J. P. (2003) "Incipient" primary hyperparathyroidism: a "forme fruste" of an old disease. *J Clin Endocrinol Metab*, 88(11), 5348-52.

Silverberg, S. J., Clarke, B. L., Peacock, M., Bandeira, F., Boutroy, S., Cusano, N. E., Dempster, D., Lewiecki, E. M., Liu, J. M., Minisola, S., Rejnmark, L., Silva, B. C., Walker, M. D. & Bilezikian, J. P. (2014) Current issues in the presentation of asymptomatic primary hyperparathyroidism: proceedings of the Fourth International Workshop. *J Clin Endocrinol Metab*, 99(10), 3580-94.

Silverberg, S. J., Lewiecki, E. M., Mosekilde, L., Peacock, M. & Rubin, M. R. (2009) Presentation of asymptomatic primary hyperparathyroidism: proceedings of the third international workshop. *J Clin Endocrinol Metab*, 94(2), 351-65.

Siprova, H., Frysak, Z. & Soucek, M. (2016) PRIMARY HYPERPARATHYROIDISM, WITH A FOCUS ON MANAGEMENT OF THE NORMOCALCEMIC FORM: TO TREAT OR NOT TO TREAT? *Endocr Pract*, 22(3), 294-301.

Souberbielle, J.-C., Massart, C., Brailly-Tabard, S., Cormier, C., Cavalier, E., Delanaye, P. & Chanson, P. (2016) Serum PTH reference values established by an automated third- generation assay in vitamin D- replete subjects with normal renal function: consequences of diagnosing primary hyperparathyroidism and the classification of dialysis patients. *European journal of endocrinology*, 174(3), 315-323.

Souberbielle, J. C., Brazier, F., Piketty, M. L., Cormier, C., Minisola, S. & Cavalier, E. (2017) How the reference values for serum parathyroid hormone concentration are (or should be) established? *Official Journal of Italian Society of Endocrinology (SIE)*, 40(3), 241-256.

Tassone, F., Maccario, M., Gianotti, L., Baffoni, C., Pellegrino, M., Cassibba, S., Cesario, F., Magro, G. & Borretta, G. (2013) Insulin sensitivity in normocalcaemic primary hyperparathyroidism. *Endocrine*, 44(3), 812-4.

Temizkan, S., Kocak, O., Aydin, K., Ozderya, A., Arslan, G., Yucel, N. & Sargin, M. (2015) Normocalcemic hyperparathyroidism and insulin resistance. *Endocr Pract*, 21(1), 23-9.

Tordjman, K. M., Greenman, Y., Osher, E., Shenkerman, G. & Stern, N. (2004) Characterization of normocalcemic primary hyperparathyroidism. *Am J Med*, 117(11), 861-3.

Tordjman, K. M., Yaron, M., Izkhakov, E., Osher, E., Shenkerman, G., Marcus-Perlman, Y. & Stern, N. (2010) Cardiovascular risk factors and arterial rigidity are similar in asymptomatic normocalcemic and hypercalcemic primary hyperparathyroidism. *Eur J Endocrinol*, 162(5), 925-33.

Traini, E., Bellantone, R., Tempera, S., Russo, S., De Crea, C., Lombardi, C. & Raffaelli, M. (2018) Is parathyroidectomy safe and effective in patients with normocalcemic primary hyperparathyroidism? *Langenbeck's Archives of Surgery*, 403(3), 317-323.

Tuna, M. M., Caliskan, M., Unal, M., Demirci, T., Dogan, B. A., Kucukler, K., Ozbek, M., Berker, D., Delibasi, T. & Guler, S. (2015) Normocalcemic hyperparathyroidism is associated with complications similar to those of hypercalcemic hyperparathyroidism. *J Bone Miner Metab*.

UCLA Introduction to linear mixed models. UCLA Statistical Consulting Group.

UKBiobank Available online: https://www.ukbiobank.ac.uk [Accessed.

Underbjerg, L., Sikjaer, T., Mosekilde, L. & Rejnmark, L. (2013) Cardiovascular and renal complications to postsurgical hypoparathyroidism: a Danish nationwide controlled historic follow-up study. *J Bone Miner Res*, 28(11), 2277-85.

Underbjerg, L., Sikjaer, T., Mosekilde, L. & Rejnmark, L. (2014) Postsurgical hypoparathyroidism--risk of fractures, psychiatric diseases, cancer, cataract, and infections. *J Bone Miner Res*, 29(11), 2504-10.

Underbjerg, L., Sikjaer, T., Mosekilde, L. & Rejnmark, L. (2015) The Epidemiology of Nonsurgical Hypoparathyroidism in Denmark: A Nationwide Case Finding Study. *J Bone Miner Res*, 30(9), 1738-44.

Van Aelst, S. & Rousseeuw, P. (2009) Minimum volume ellipsoid. *Wiley Interdisciplinary Reviews: Computational Statistics*, 1(1), 71-82.

Vautour, L. & Goltzman, D. (2018) Regulation of Calcium Homeostasis. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, 163-172.

Venkat, K. K., Arora, M. M., Singh, P., Desai, M. & Khatkhatay, I. (2009) Effect of alcohol consumption on bone mineral density and hormonal parameters in physically active male soldiers. *Bone*, 45(3), 449-454.

Vignali, E., Cetani, F., Chiavistelli, S., Meola, A., Saponaro, F., Centoni, R., Cianferotti, L. & Marcocci, C. (2015) Normocalcemic primary hyperparathyroidism: a survey in a small village of Southern Italy. *Endocr Connect*, 4(3), 172-8.

Wade, T. J., Yen, T. W., Amin, A. L. & Wang, T. S. (2012) Surgical management of normocalcemic primary hyperparathyroidism. *World J Surg*, 36(4), 761-6.

Walsh, J., Gittoes, N. & Selby, P. (2016) SOCIETY FOR ENDOCRINOLOGY ENDOCRINE EMERGENCY GUIDANCE: Emergency management of acute hypercalcaemia in adult patients.

Wang, X., Meng, L., Su, C. & Shapses, S. A. (2019) LOW FREE BUT NOT TOTAL 25-HYDROXYVITAMIN D LEVELS IN SUBJECTS WITH NORMOCALCEMIC HYPERPARATHYROIDISM. *Endocrine Practice*.

Wikipedia (2017) *Mahalanobis distance*, 2017. Available online: https://en.wikipedia.org/wiki/Mahalanobis_distance [Accessed.

Worcester, E. M. & Coe, F. L. (2008) New insights into the pathogenesis of idiopathic hypercalciuria. *Semin Nephrol*, 28(2), 120-32.

Yacobi-Bach, M., Serebro, M., Greenman, Y., Tordjman, K. & Stern, N. (2015) Letter to the editor: Thiazides are not inducers of PTH secretion: a comment on normocalcemic hyperparathyroidism. *J Clin Endocrinol Metab*, 100(2), L27-8.

Yener Ozturk, F., Erol, S., Canat, M. M., Karatas, S., Kuzu, I., Dogan Cakir, S. & Altuntas, Y. (2015) Patients with normocalcemic primary hyperparathyroidism may have similar metabolic profile as hypercalcemic patients. *Endocr J*.

Yu, Q., Liu, K., Ma, D., Xie, C., Wu, Y., Dai, W. & Jiang, H. (2019) Do Symptoms and Serum Calcium Levels Affect the Results of Surgical Treatment of Primary Hyperparathyroidism? *BioMed research international*, 2019, 2150159-2150159.

Section 4: Supplementary material

The R codes used for the analyses can be found in Supplementary material (included in the attached CD).