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NOVEL TECHNOLOGICAL APPLICATIONS FOR THE MANAGEMENT OF ENDOCRINE DISEASES

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NOVEL TECHNOLOGICAL APPLICATIONS FOR THE MANAGEMENT OF ENDOCRINE DISEASES

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STATEMENT OF ORIGINALITY

Unless otherwise stated, the work described in this thesis was carried out at Campus Bio-Medico University of Rome, Italy.

The author designed the studies that are reported in this thesis and/or analyse and described the results.

I hereby state that this thesis entitled "**Novel technological applications for the management of endocrine diseases**" has not been submitted for a degree or any other qualification at any other university.

Anda Mihaela Naciu, June 2021

1. RESEARCH QUESTION

What is the prevalence of cardiovascular autonomic neuropathy in subjects affected by postsurgical hypoparathyroidism? Is there an association between hypocalcemia, low PTH levels, hyperphosphatemia and cardiovascular autonomic neuropathy?

ABSTRACT

Introduction: Postsurgical hypoparathyroidism (hypoPT) increases fatigue and seems to affect the risk of mortality. Cardiovascular autonomic neuropathy (CAN) is an impairment of the cardiovascular autonomic system, a cause of increased mortality, and associated with increased fatigability. The aim of this study is to evaluate CAN in hypoPT and its relationship with hypocalcemia, PTH levels, and hyperphosphatemia.

Methods: This is a cross-sectional study comparing 51 postsurgical hypoPT patients treated with calcium and calcitriol and 43 control subjects without any PTH/calcium/phosphate disorders who underwent thyroidectomy. CAN was assessed by heart rate (HR) response to deep breathing, HR response to the lying-to-standing test, HR response to the Valsalva maneuver, and blood pressure response to standing. Participants were considered to have "early CAN" if they had one abnormal result in the HR tests and "definite CAN" with two or more abnormal results.

Results: The prevalence of CAN was 23% in the control group and 78% in the hypoPT group (OR 11.48; 95% CI, 4.48 to 32.17). Patients with hypoPT and serum calcium (sCa) 8.5 mg/dL had a prevalence of early CAN of 72.4% and the prevalence was 86.4% in those with sCa <8.5 mg/dL. Definite CAN was found in 2.3% of the control group, 24.1% of the hypoPT group without hypocalcemia, and 59.1% of the hypoPT group with hypocalcemia. In the hypoPT group, the OR for definite CAN in the patients with hypocalcemia compared to the patients with normocalcemia was 4.54 (95% CI, 1.36 to 15.11). The association between low sCa and definite CAN was confirmed after adjustment for confounders with OR 13.62 (95% CI, 2.12 to 149.84). No association was found between definite CAN and PTH levels or high phosphate levels.

Conclusions: HypoPT is associated with CAN and hypocalcemia seems to affect its severity. Larger and prospective studies are needed to confirm our findings.

2. RESEARCH QUESTION

Can cardiovascular autonomic neuropathy explain fatigue in subjects affected by postsurgical hypoparathyroidism?

ABSTRACT

Introduction: Hypoparathyroidism (hypoPT) results in an impairment of quality of life (QoL), an increase in fatigue and a higher risk of mortality. Cardiovascular autonomic neuropathy (CAN) is an impairment of the cardiovascular autonomic system and is associated with increased mortality and fatigability. Patients with hypoPT show an increased risk of CAN. However, no previous studies have investigated the association between CAN and QoL in hypoPT. To test whether CAN is associated with fatigue and impaired QOL in hypoPT patients.

Methods: We enrolled 48 subjects with postsurgical hypoPT treated with calcium and calcitriol and 38 healthy subjects who underwent thyroidectomy. Subjects completed the RAND 36-Item Short Form (SF-36) Health Survey, evaluating physical (PCS) and mental (MCS) health, and fatigue score. CAN was assessed using cardiovascular autonomic reflex tests (CARTs). Participants were considered to have "early CAN" (EC) if they had one abnormal CART and "definite CAN" (DC) with two or more abnormal CARTs.

Results: Compared with controls, hypoPT population had lower fatigue scores (44.5 IQR:9 vs 38.5 IQR:12.3, P = 0.031). In the hypoPT group, only participants with DC had a lower fatigue score than subjects without CAN (DC: β: -9.55, P = 0.005) after adjusting for age, duration of disease, calcium concentration, TSH, calcitriol and calcium supplementation. No differences were found in the PCS and MCS scores in the hypoPT group.

Conclusions: CAN may explain fatigue, a common complaint of postsurgical hypoPT patients. Further larger and prospective investigations are needed to confirm our findings.

3. RESEARCH QUESTION

How is the bone quality assessed by bone strain index in subjects affected by primary hyperparathyroidism?

ABSTRACT

Introduction: Primary hyperparathyroidism (PHPT) is associated with impaired bone quality and increased fracture risk. Reliable tools for the evaluation of bone quality parameters are not yet clinically available. Bone Strain Index (BSI) is a new metric for bone strength based on Finite Element Analysis from lumbar spine and femoral neck dual X-ray absorptiometry images. We aimed to investigate the lumbar spine (LS), femoral neck (FN), and total hip (TH) BSI in PHPT compared to controls.

Methods: In this cross-sectional study we assessed the LS-BSI, FN-BSI and TH-BSI in 44 PHPT and 39 age- and sex-matched control subjects.

Results: TH bone mineral density (BMD) and 1/3 distal radius BMD were lower in the PHPT group than in controls (TH 0.802±0.13 vs 0.872±0.09, P<0.05; radius 0.565±0.07 vs 0.620±0.06, P<0.001). There were no differences between groups in trabecular bone score (TBS) and T-score adjusted for TBS. BSI was significantly higher at LS (2.20±0.58 vs 1.94±0.48, p=0.003), FN (1.66±0.39 vs 1.40±0.36, p=0.003) and TH (1.46±0.3 vs 1.24 ±0.25, p=0.001) in PHPT. LS-BSI showed moderate accuracy for detecting VFx [(area under the ROC curve 0.68 (CI:0.52-0.848)]. The best cut-off was set at 2.12 (sensibility 72%, specificity 64%, accuracy 67.4%).

Conclusions: BSI, a DXA-derived bone quality index, is impaired in PHPT and may help to identify PHPT subjects at high risk of fractures.

4. RESEARCH QUESTION

How is the bone quality assessed by trabecular bone score and how this tool may be able to detect vertebral fractures in subjects with normocalcemic hyperparathyroidism?

ABSTRACT

Introduction: the impact of normocalcemic hyperparathyroidism (NHPT) on bone quality remains largely unexplored. We aimed to investigate the usefulness of trabecular bone score (TBS) assessment in NHPT and the accuracy of TBS in detecting vertebral fractures (VFs) in NHPT.

Methods: In this multicentric cross-sectional study, we assessed the TBS in 47 subjects with NHPT, 41 with primary hyperparathyroidism (PHPT) and 39 age- and sex-matched control sub-jects.

Results: TBS values did not differ among the three groups. The prevalence of low TBS (TBS<1.2) was 23.4% in NHPT, 26.8% in PHPT and 15.4% in controls, without statistically significant differences between groups. However, we found a lower Lumbar spine Z-score adjusted for TBS (LS Z-score*TBS) in PHPT participants when compared with controls (-0.48 ± 1.06 vs 0.07 ± 0.93, p: 0.017). In NHPT group, LS Z-score*TBS did not detect patients with overall VFs (Threshold -0.15, AUC 0.45 95%CI 0.253-0.648, accuracy 55.3%). Instead, it was useful for moderate-severe VFs (Threshold 0.55, AUC 0.81, 95%CI 0.62-0.996, accuracy 83%). In PHPT subjects also, TBS did not identify patients with VFs.

Conclusions: In NHPT, TBS is not reduced. When adjusted for TBS, the LS Z-score does predict moderate to severe VFs.

STATEMENT OF ATTRIBUTION

1) Author's contributions for the following trial: **"*Cardiovascular autonomic neuropathy as a new complication of postsurgical chronic hypoparathyroidism"***.

Conceived and designed the trial: Gaia Tabacco, Anda Mihaela Naciu, Claudio Pedone and Andrea Palermo. *Performed the trial*: Gaia Tabacco, Anda Mihaela Naciu, Daria Maggi, Assunta Santonati, Roberto Cesareo, Daniela Bosco, Gianluigi Gaspa, Nicola Napoli, Paolo Pozzilli, Silvia Manfrini and Andrea Palermo. *Analyzed and interpretated the data*: Gaia Tabacco, Anda Mihaela Naciu, Claudio Pedone and Andrea Palermo. *Wrote the manuscript:* Gaia Tabacco, Anda Mihaela Naciu and Andrea Palermo.

2) Author's contributions for the following trial: **"*Cardiovascular autonomic neuropathy as a cause of fatigue in chronic hypoparathyroidism"***.

Conceived and designed the trial: Gaia Tabacco and Andrea Palermo. *Performed the trial*: Gaia Tabacco, Anda Mihaela Naciu, Daria Maggi, Roberto Cesareo, Assunta Santonati, Daniela Bosco, Alfonso Maria Di Tommaso, Paolo Pozzilli, Silvia Manfrini and Andrea Palermo. *Analyzed and interpretated the data*: Gaia Tabacco, Anda Mihaela Naciu, Claudio Pedone, Diana Lelli and Andrea Palermo. *Wrote the manuscript:* Gaia Tabacco, Anda Mihaela Naciu and Andrea Palermo.

3) Author's contributions for the following trial: **"*Dxa-based bone strain index: a new tool to evaluate bone quality in primary hyperparathyroidism"*** *(manuscript under revision)*.

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and Andrea Palermo. *Analyzed and interpretated the data*: Gaia Tabacco, Anda Mihaela Naciu, Carmelo Messina, Luca Rinaudo, Roberto Cesareo, Stefania Falcone, Silvia Manfrini, Nicola Napoli, John P Bilezikian, Fabio M Ulivieri and Andrea Palermo. *Wrote the manuscript:* Gaia Tabacco, Anda Mihaela Naciu, Carmelo Messina, John P Bilezikian, Fabio M Ulivieri and Andrea Palermo.

4) Author's contributions for the following trial: **"*Bone quality, as measured by trabecular bone score in normocalcaemic primary hyperparathyroidism*"** *(manuscript under revision)*.

Conceived and designed the trial: Anda Mihaela Naciu, Gaia Tabacco, Roberto Cesareo and Andrea Palermo. *Performed the trial*: Anda Mihaela Naciu, Gaia Tabacco, Stefania Falcone, Daria Maggi, Nicola Napoli, Silvia Manfrini, Roberto Cesareo and Andrea Palermo. *Analyzed and interpretated the data*: Anda Mihaela Naciu, Gaia Tabacco, Iacopo Chiodini, Claudio Pedone, Diana Lelli, John P Bilezikian, Roberto Cesareo and Andrea Palermo. *Wrote the manuscript:* Anda Mihaela Naciu, Gaia Tabacco, Iacopo Chiodini, John P Bilezikian, Roberto Cesareo and Andrea Palermo.

ABBREVIATIONS

APS1: autoimmune polyglandular syndrome

ATP : adenosine triphosphate

BMI: body mass index

BMD: bone mineral density

BSI: bone strain index

CAN: cardiovascular autonomic neuropathy

CaSR: Ca^{2+}sensing receptor

CKD-EPI: Chronic Kidney Disease–Epidemiology Collaboration

CTAL: cortical thick ascending limb

CTX: C-terminal crosslinking telopeptides of type I collagen

ECF: extracellular fluid

eGFR: estimated glomerular filtration rate

ER: endoplasmic reticulum

FEM: finite element method

FGF23: fibroblast growth factor 23

FHH: familial hypocalciuric hypercalcemia

FN: femoral neck

GATA3: GATA binding protein 3

GCMB: glial cell missing

GI: gastro-intestinal

GNA11: G-protein alpha subunit 11

hypoPT: hypoparathyroidism

HR: heart rate

HRpQCT: high-resolution peripheral quantitative computed tomography

LS: lumbar spine

MCS: mental component summary

MEN: multiple endocrine neoplasia

microCT: micro-computed tomography

NHERF1: sodium-hydrogen exchanger regulatory factor-1

NHPT: normocalcemic hyperparathyroidism

NPT2a: sodium-coupled transporters a

NPT2c: sodium-coupled transporters c

OC: osteocalcin

P1NP: N-terminal crosslinking propeptides of type I procollagen

PCS: physical component summary

PHPT: primary hyperparathyroidism

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PTH: parathyroid hormone

PTHR1: PTH receptor

PTHrP: PTH-related peptide

PTX: parathyroidectomy

QOL: quality of life

SF-36: 36-Item Short Form Health Survey

SHPT: secondary hyperparathyroidism

TBS: trabecular bone score

TH: total hip

TRAP-5b: tartrate-resistant acid phosphatase 5b

TRPM7: transient receptor potential cation channel subfamily M member 7

TRPV6: transient receptor potential vanilloid type 6

TSH: thyroid-stimulating hormone

vBMD: volumetric BMD

VDR: vitamin D receptor

VFA: vertebral fracture assessment

VFx: vertebral fractures

VRE: vitamin D response element

25(OH)D3: 25-hydroxyvitamin D3

1,25(OH)2D3: 1,25-dihydroxyvitamin D3

CHAPTER 1

GENERAL BACKGROUND

1.1 Calcium

In adults, almost all calcium (99%) is located in the skeleton as hydroxyapatite crystal [Ca10(PO4)6(OH)2], that is responsible for the mechanical weight-bearing properties of bones. The crystal represents as a ready source of calcium to sustain some calcium-dependent biological systems and to preserve blood ionized calcium within the normal range. The remaining 1% represents the "non-bone" calcium, that is contained in the blood, extracellular fluid, and soft tissues. It takes part in various fundamental biological processes inside the body. Around 50% of this "non-bone" calcium consists of ionized fraction (the biologically functional portion of total calcium that can be measured clinically), while 40% is linked to proteins (mainly albumin) and 10% is complexed to ions (i.e. calcium phosphate, calcium carbonate, and calcium citrate) (1,2). Non-bone calcium is essential for vascular and muscle functions, intracellular signaling, nerve transmission and hormonal release. Bone calcium is a container for all these metabolic requirements due to the bone remodeling process (3).

Regarding cellular system, cytosol calcium is 10−6 M, which creates a 1,000- fold gradient through the plasmatic membrane [extracellular fluid (ECF) calcium is 10−3M] that promotes calcium entry in the cell. The electrical charge across the plasmatic membrane is about 50mV with the cell interior negative. In this way, the chemical and electrical gradients through the plasmatic membrane support calcium entry, which the cell must defend against to preserve cell survival (1). Calcium-induced cell death is widely avoided by numerous mechanisms including calcium extrusion from the cell by adenosine triphosphate (ATP)- dependent energy driven calcium pumps and channels; sodium– calcium exchangers; and the association of intracellular calcium with proteins located in the cytosol, endoplasmic reticulum (ER), and mitochondria. Calcium linked to ER and mitochondrial sites buffer intracellular calcium and can be mobilized to keep

cytosol calcium rates and to generate pulsatile peaks of calcium to arbitrate membrane receptor signaling that adjust several biologic systems.

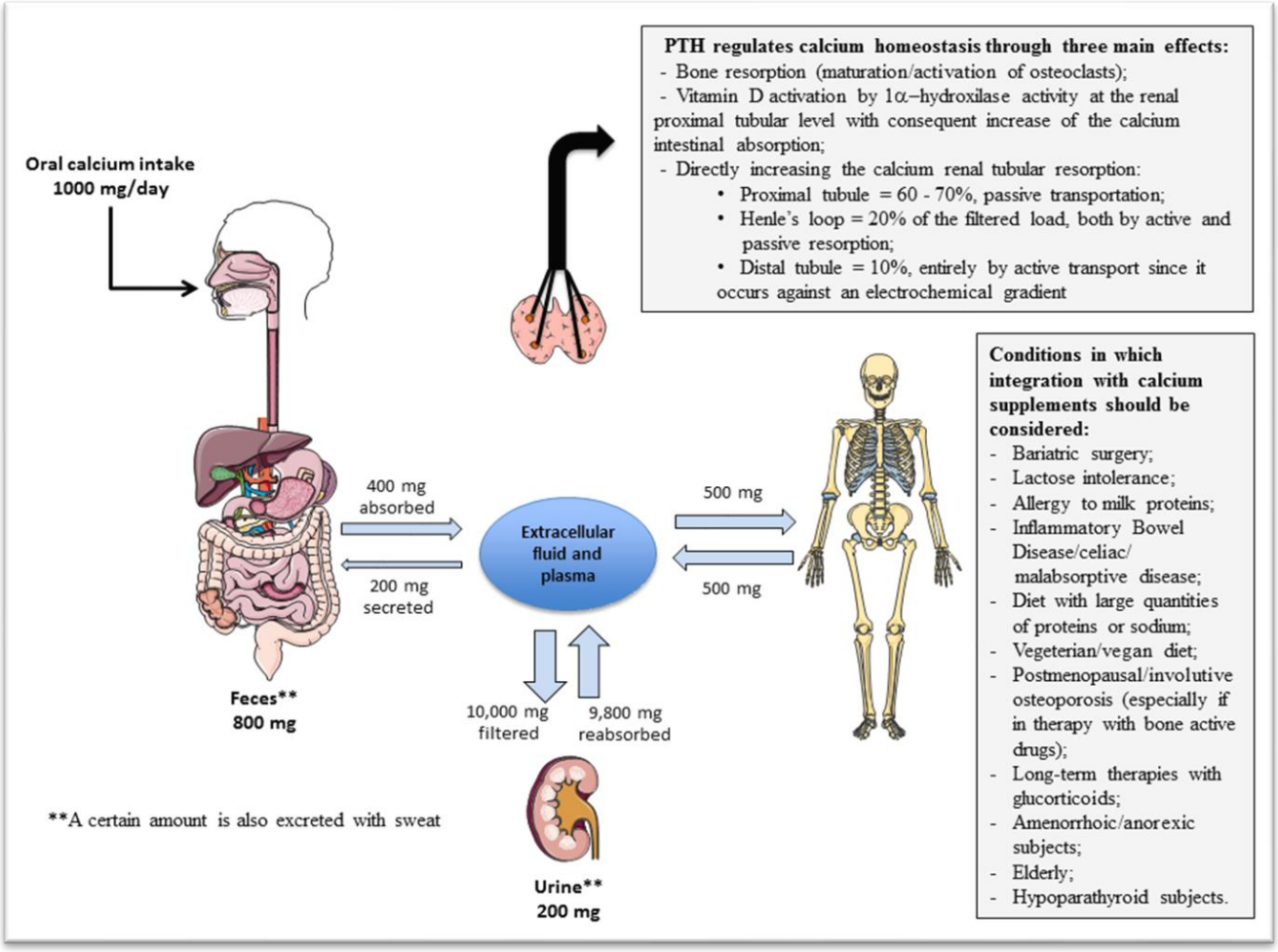
Even if only the ionized calcium is able to move into cells and activates numerous cellular mechanisms, several clinical laboratories determine total serum calcium levels. Concentrations of total serum calcium in healthy subjects usually range between 8.5 and 10.5 mg/dL (2.12 to 2.62 mmol/L). The normal levels of ionized calcium are 4.65–5.25mg/dL (1.16–1.31 mmol/L). When protein concentrations (in particular albumin) oscillate, total calcium levels may change, instead the ionized calcium (whose level is hormonally regulated) remain quite stable. Some conditions like dehydration/haemoconcentration may elevate serum albumin and others with decreased albumin levels may not accurately reflect serum calcium concentrations. This is the reason why is important to "correct" the total calcium level by subtracting 0.8 mg/dL from the total calcium for every 1.0 g/dL by which the serum albumin level is greater than 4 g/dL. Instead, if albumin concentrations are low, total calcium should be corrected using this formula: corrected calcium (mg/ dL) = measured total calcium (mg/dL) + 0.8 (4.0 – serum albumin [g/dL]). Furthermore, blood pH changes may alter the albumin–Ca2+ complex balance, with acidosis decreasing the binding and alkalosis enhancing it. Fluctuations in serum protein or pH need determination of the ionized calcium concentration to establish the physiologic serum calcium level. The ECF calcium level must be preserved within a rather narrow range because calcium regulates numerous cellular mechanisms (4).

The diet represents the main source of calcium and currently the dietary recommended allowance is between 700 and 1200 mg/day (5). The "fractional calcium absorption" accounts the rate of calcium absorbed by the gut, and it seems to be around 20-30% of the total intake (6). In fact, in healthy young adults, 200 mg out of 1000 mg of daily consumed calcium will be absorbed and it will be part of the exchanging pool (plasma, bone, ECF, cells). Mainly urinary excretion and to a smaller extent sweat, skin, and hair, constitute the calcium exit ways from this pool. The calcium not absorbed in the intestine is excreted in the faeces (75%) (7). The close regulation of calcium absorption

and excretion keeps the extracellular ionized calcium in the normal range concentrations while allowing the exchange of calcium to and from essential stores (2).

The skeleton represents the major storage site for calcium in the body (1000 g of calcium). Moreover, a normal bone turnover provides about 500 mg of calcium to blood from the bone, instead the same amount of calcium is captured by bone from the blood. The gut, the skeleton and the kidney carry out an important role in guaranteeing calcium homeostasis (Figure 1).

Figure 1. Calcium homeostasis and main conditions that need calcium supplementation(8)



The maintenance of the ECF calcium concentrations is provided through the action of calcium-sensitive cells that control the hormone productions (9). In fact, they act on

specific cells (bone, kidney and bowel) which can react by altering fluxes of calcium to preserve ECF calcium levels. In particular, a decrease in ECF calcium sensed by Calcium Sensing Receptor (CaSR) at the parathyroid gland level, stimulates parathyroid hormone (PTH) release. PTH-mediated bone resorption liberates both calcium and phosphate from the skeleton, while PTH stimulates calcium reabsorption in the kidney and, at the same time, inhibits phosphate reabsorption increasing phosphaturia. Both hypocalcemia and PTH elevation are able to induce the conversion of the 25-hydroxyvitamin D3 (25(OH)D3), to the active form of vitamin D 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (9), that stimulates the intestinal absorption of calcium. This integrated hormonal mechanism restores serum calcium and closes the negative feedback loop. Once concentrations of serum calcium increase, all these mechanisms are reversed, with the restoration of normal serum calcium levels (2,10).

1.1.1 Intestinal calcium transport

Net intestinal calcium absorption can be measured by the external balance technique in which the amount of calcium ingested is known, and urine calcium excretion and fecal calcium loss are measured. Negative absorption happens when net absorption diminishes to about 200 mg calcium per day (5.0 mmol). The quantity of dietary calcium absorbed varies with age, amount of calcium ingested and may range from 20% to 60%. Some conditions such as growing children, during growth spurts in adolescence, pregnancy and lactation are associated with higher rates of net calcium absorption. During prolonged dietary calcium restriction, the efficiency of calcium absorption is higher, in order to absorb the greatest portion of calcium ingested. Net absorption decreases with age in both men and women, and an increased calcium intake is required to cover the lower absorption rate. Fecal calcium losses range from 100 to 200 mg per day (2.5 to 5.0mmol). Fecal calcium consists in unabsorbed dietary calcium and calcium from intestinal, pancreatic, and biliary secretions. Secreted calcium is not adjusted by hormones or serum calcium. In the large surface area of the duodenum and

jejunum about 90% of calcium is absorbed. Increased calcium demand promotes expression of the epithelial calcium active transport system in the duodenum, ileum, and throughout the colon able to improve fractional calcium absorption in older men and women from 20% to 45% and in children and young adults from 20% to 55%-70%. The efficiency of the small intestine and colon to absorb alimentary calcium is increased by 1,25(OH)2D3. Active calcium absorption can achieve about 10–15% of a dietary load. Reduced dietary calcium intake can increase PTH secretion and 1,25(OH)2D3 production. 1,25(OH)2D3 regulates calcium absorption that involves the entrance of calcium into the enterocyte using the apical calcium channel, namely the transient receptor potential vanilloid type 6 (TRPV6). Once in the cell, it has been suggested that the calcium-binding protein calbindin-D9k facilitates the diffusion of calcium in the cytoplasm until it is released at the basolateral side through the intestinal plasma membrane pump PMCA1b (11).

Intestinal epithelial calcium transport includes two mechanisms, an energy-dependent, cell-mediated saturable active process that is controlled by 1,25(OH)2D3, and a passive, diffusional paracellular path of absorption that is regulated by transepithelial electrochemical gradients. The cell-mediated pathway involving the TRPV6 calcium channel is saturable with a Kt (1/2 maximal transport) of 1.0 mmol. Passive diffusion is not saturable and is dependent on luminal calcium concentrations. In adults fed a diet low in calcium, enhanced 1,25(OH)2D3 production regulate the paracellular calcium diffusion by increasing tight junctions permeability to the ions. High dietary calcium intake absorption determine 1,25(OH)2D3 suppression, and passive paracellular transport accounts for most all absorption (12).

1.2 PTH and mineral homeostasis

PTH is a major systemic calcium-regulating hormone and an important regulator of bone and mineral homeostasis. There can be severe clinical consequences when the extracellular levels of calcium and phosphorus deviate above or below their normal

targets, so the availability of homeostatic systems ensuring maintenance of their circulating concentrations within the respective normal ranges is critical (13,14). The parathyroid cell's capacity to detect small (≈ 1–2%) variations in Ca^{2+} from its normal range and to restore normocalcemia with appropriate alterations in the secretion of PTH plays an important role. The sensitivity of the parathyroid glands to variations in Ca^{2+} is a consequence of the steep inverse sigmoidal relationship between PTH release to Ca^{2+} (15). PTH restores serum calcium by different mechanisms: (i) release of calcium and phosphorus from the bones through stimulation of osteoclastic activity (16,17); (ii) increases renal synthesis of 1,25(OH)2 D3 from 25(OH)D3 in the proximal tubule with increase dietary absorption of calcium and phosphorus in the gut; (iii) decrease in calcium excretion and a concomitant decrease in phosphate reabsorption in the kidney (18); and (iv) promotes renal phosphate excretion, an action that can contribute to excretion of any excess phosphate arising from increased gastrointestinal (GI) absorption and/or net release from bone. The hypo- and hyperphosphatemia that can be founded in subjects with hyper- or hypoparathyroidism (19), respectively, speak to the physiologically significant role of PTH in maintaining phosphate homeostasis.

The rate of synthesis and release of PTH is increase by chronic decreases in serum calcium (20). Other factors such as epinephrine, calcitonin, vitamin D, magnesium, and phosphate impact the synthesis and release of PTH (21). The CaSR acting as a sensor for ionized calcium concentrations provides the relationship between circulating ionized calcium levels and PTH secretion, keeping calcium within a narrow range. High extracellular calcium concentrations sensed by the CaSR determine a reduction of PTH secretion and an increase of Ca++ excretion by the kidney (22). Conversely, lower concentrations of plasma calcium stimulate PTH secretion and Ca++ reabsorption by the kidney (22).

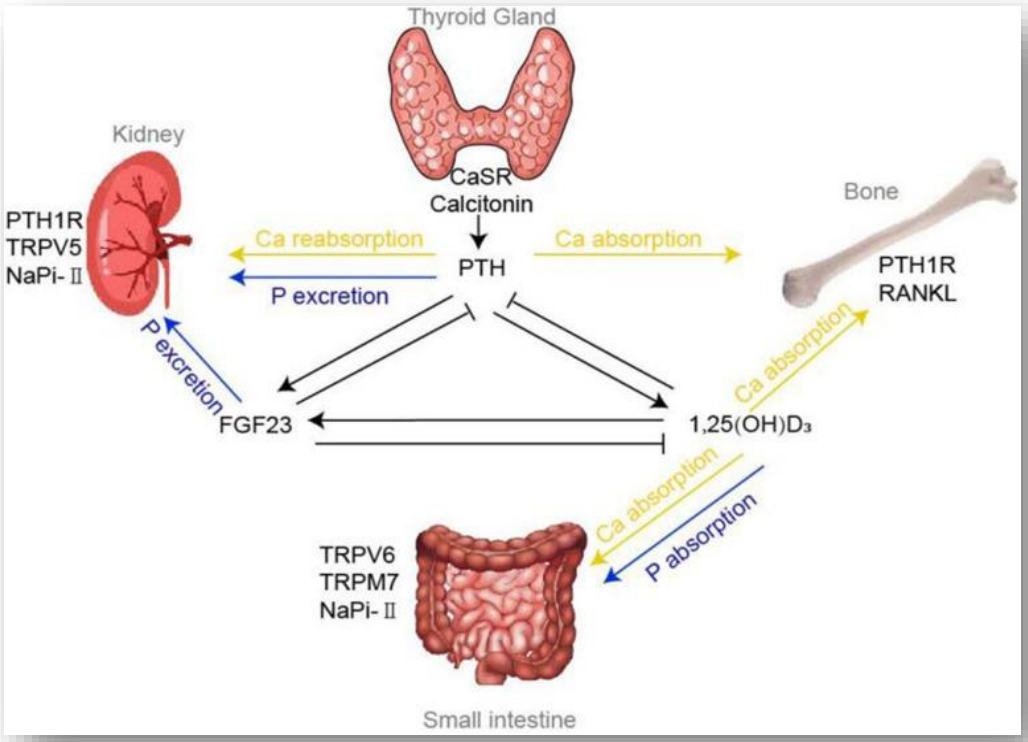
About 20% to 30% of PTH secretion is pulsatile and its concentrations presents relevant fluctuations during the course of the day (23). The circadian rhythm for PTH reaches a more pronounced peak in the early morning, a nadir in the late morning and a second, lower peak in the afternoon, likely related to calcium, phosphate and calcitriol

changes which can be modified by nutritional intake of these elements (23). Up to now, the mechanisms for the pulsatile secretion of PTH are not very well investigated, nor are the functional consequences.

The parathyroid glands have the capacity to increase their mass by 10–100-fold or more during chronic hypocalcemia, so-called secondary hyperparathyroidism (SHPT), especially in patients with chronic renal insufficiency. The time course of these different adaptive changes, that can last from seconds to indefinite duration, ensure that there is a rapid response, but also elements of the response that persist for as long as is needed, without any "windows" where an enhanced secretory response is not present.

Over the last years, however, another homeostatic system important seems to play an important role for maintaining phosphate homeostasis and, to a lesser extent, Ca^{2+} homeostasis. Central to this system is the phosphaturic hormone, fibroblast growth factor 23 (FGF23), which is secreted by osteocytes, osteoblasts that have become encased within bone during bone formation and seems to have an inhibitory effect on PTH production and secretion during normocalcemia. During hypocalcemia, when increased PTH secretion is needed to restore the normal calcium levels, this inhibitory effect of FGF23 seems to be abolished (24). FGF23 also engages in cross-talk with the hormones that maintain Ca^{2+} homeostasis. FGF23 has a potent phosphaturic effect on the kidney (inhibitory role on NaPi-II cotransporters) and also inhibits the renal production of 1,25(OH)2D3, thereby reducing absorption and release of phosphate from intestine and bone, respectively. Since both hyperphosphatemia and a decrease in 1,25(OH)2 D3 stimulate PTH secretion, the increase in PTH in addition to FGF23 elevation, further increase the renal phosphate wasting. The identification of the vital TRPV, Transient Receptor Potential Cation Channel Subfamily M Member 7 (TRPM7), vitamin D receptor (VDR), NaPi cotransporters, and CaSR all have further advanced our understanding of the direct and indirect interactions between FGF23 and PTH (Figure 2) (25).

Figure 2. Calcium and phosphorus homeostasis (25).



1.3 PTH action on skeleton

1.3.1 Anabolic effect

Studies on PTH "knock-out" mice have shown that the lack of PTH leads to a reduction in metaphyseal osteoblasts and a decrease in trabecular bone in fetal and neonatal mice. These findings are in according with a significant physiologic role of PTH in promoting the development of the fetal and newborn skeleton, and consequently in bone anabolism (26). This anabolic effect in early growth may be observed later in life as reflected by the fact that endogenous PTH also looks appropriate for better fracture healing in mice (27). In patients with primary hyperparathyroidism (PHPT), bone densitometry demonstrates that trabecular bone in sites such as the vertebrae is maintained whilst elevated levels of circulating PTH. These results are proved by examinations of bone biopsy specimens by micro-computed tomography (microCT).

The overall findings shown preserved trabecular bone volume and microarchitecture in most patients with PHPT (28). Exogenous PTH demonstrated to increase fracture repair in animals (29) and its role may be moderated by endogenous rates of the hormone (27). Whilst the robust evidence from animal experiments reported that PTH can improve normal fracture healing, this effect has still not been demonstrated beyond a reasonable doubt in humans. Systemic anabolic agents including PTH and PTH-related protein (PTHrP) have demonstrated potential as candidates that could increase localized osseous healing, when administered intermittently (30–32). Clinical studies using exogenous PTH(1–34) (31) or PTH(1–84) (33) in subjects with osteoporosis have demonstrated that both molecules improve bone mineral density (BMD) and bone microarchitecture. BMD improvement occur preponderant in areas rich in trabecular bone. The enhancement in trabecular bone volume, number and connectivity, as well as increases in plate-like relative to rod like structures, have been demonstrated by microCT examinations of iliac crest biopsies (34). PTH(1–34) demonstrated to increase bone formation rates in iliac crest biopsies, on the trabecular, endosteal, and periosteal surfaces. The greatest effects on trabecular bone seem to be expressed in the reduction of vertebral fractures (VFx) described with both PTH(1–84) and PTH(1–34). PTH(1–34) has also demonstrated to diminish extra-VFx (31), probably due to its impact on increasing periosteal apposition, leading to higher periosteal width, and then improved long bone strength. The intermittent administration of PTH in subjects with osteoporosis has shown that there is an early phase of stimulation of bone formation without resorption, which is followed subsequently by stimulation of bone resorption (35). The phase during which the impact of PTH on bone formation relative to resorption are maximal has been called the "anabolic window" (36). The magnitude of the PTH anabolic response seems to be due to the extent of the remodeling space, as measured by the number and activity of osteoclasts on bone surfaces (37). This concept is in accordance with the observation that there is reduced ability of PTH to perform its anabolic role after treatment with bisphosphonates (38,39) or after targeted deletion of the c-fos proto-oncogene, which is known to be crucial for osteoclastogenesis (40).

Globally, both animal and human evidence of endogenous PTH deficiency or excess, or of exogenous PTH use, offer convincing evidence that PTH has an important role for normal trabecular bone quantity and quality and that important improvement in periosteal apposition may occur, which may also be favorable.

PTHrP, which binds to the same receptor (PTH1R) as PTH and has similar bioactivity in their N-terminal region, plays a crucial role in normal development of the growth plate. PTHrP was initially discovered in the search for a PTH-like factor that determine hypercalcemia of malignancy (41). In osteoporosis clinical studies, PTHrP(1–36) has been demonstrated to be a potent anabolic product, raising the likelihood that NH2 - terminal forms of PTHrP will be efficient after optimal analogs and doses have been defined. In studies that involved postmenopausal osteoporosis and had fracture risk as an outcome, PTHrP(1–36) significantly reduced fracture risk and increased trabecular bone scores (32).

1.3.2 Catabolic effect

Although PTH exerts a physiologic role to preserve trabecular bone mass in the fetus and also in the neonate, by 4 months of age PTH-null mice on a normal diet exhibit increased rather than reduced bone mass, in particular in cortical bone (42). This is in accordance with decreased bone resorption normally done by PTH to preserve normocalcemia. Subsequently, in the postnatal environment of the older animals, where external calcium fonts are different from those in the fetus and neonate and where control of calcium homeostasis varies, PTH seems to protect against a reduction in serum calcium by improving bone resorption higher than formation. In subjects with hypoparathyroidism, increased cortical bone has also been reported after measurement by bone densitometry (43). On the other hand, in PHPT, preferred decrease of cortical relative to trabecular bone has been reported by bone densitometry and examination of bone biopsy specimens by microCT. Thus, in most cases of subjects with PHPT cortical thickness is decreased and increased cortical porosity is noted. BMD loss may therefore be noted frequently in areas of increased cortical bone such as the distal third

of the radius and to a minor part in the hip. Some subjects, usually with a more severe form of PHPT, may also present reductions in bone mass at lumbar spine and an unusual trabecular bone compartment with decreased trabecular number and volumetric density and increased trabecular spacing (44). In advanced forms, generalized bone loss, localized brown tumors (osteitis fibrosa cystica), bone cysts, and sub-periosteal resorption of phalanges or the distal clavicle may happen, all compatible with a bone remodeling pronouncedly increased. In the more advanced forms of PHPT fracture risk may be augmented but whether this happens in the milder forms is not certain. Globally, therefore, these and other (45) animal and human trials provide robust findings that PTH determine a preservation of trabecular bone and diminished cortical bone, at least until very high levels of circulating PTH are expected when more generalized bone resorption may happen to release calcium from skeletal stores.

1.4 PTH and kidney

The kidneys are a principal target of PTH action, where it adjusts calcium and phosphate transport, vitamin D biosynthesis and degradation, and intermediary metabolism. The enduring and insightful investigations of Fuller Albright and his research team demonstrated the principal effect of PTH to determine a rapid increase of phosphate excretion and plasma calcium levels. These elementary studies of mineral ion balance served as a foundation for the robust work that followed on cell and molecular interplays.

1.4.1 PTH and phosphate

PTH supports calcium and magnesium absorption, while inhibits phosphate, bicarbonate, sodium and potassium absorption. The most important and first effect of PTH to be detected in the kidney was the inhibition of phosphate absorption. Renal phosphate transport is mainly limited to proximal tubules, where two sodium-coupled transporters NPT2a (SLC34A1)1 and NPT2c (SLC34A3) arbitrate uptake of phosphate from luminal fluid. PTH determine renal phosphate transport inhibition by the

endocytic retrieval and metabolic down-regulation of brush border NPT2a and, to a minor extent, NPT2c. Latest evidences suggest that continuous exposure to PTH not only down-regulates NPT2a expression but also decrease NPT2a mRNA stability (46). PTH supports endocytosis of NPT2a and NPT2c in polarized OK cells and in isolated tubule preparations. Remarkably, apical consequences of PTH are prevalently mediated by a phospholipase C (PLC) and protein kinase C (PKC) signaling mechanism, while basolateral PTH impact seem principally to occurs through the adenylyl cyclase and protein kinase A pathway (PKA). In mouse proximal tubules, PKA clamping abolishes dopamine-inhibitable phosphate transport. Consequently, PKA activation appear to be necessary and adequate for the acute impact of PTH on phosphate transport (47). PTH significantly mediates targeted phosphorylation of sodium-hydrogen exchanger regulatory factor-1 (NHERF1), which later increases bound Npt2a to promote internalization and so inhibit phosphate transport (48). When NHERF1 is absent, apical NPT2a expression is reduced, cytoplasmic protein accumulation is increased with a correspondingly elevation of urinary phosphate excretion (49). The mechanism by which NHERF1 mediates phosphate transport is more intricate than previously described. It is not well defined how apical and baso-lateral signals resulting from PTHR1 activation are complemented to maintain spatiotemporal coordination of phosphate transport.

1.4.2 PTH and calcium

The renal tubule reabsorbs approximately 97.5% of calcium. Indeed, the majority of calcium recovery (65%) happens in proximal tubules (50). However, PTH exerts its effect on calcium absorption at distal nephron sites including cortical thick ascending limbs, distal convoluted and connecting tubules. Calcium transport in proximal tubules is mostly thermodynamically passive, proceeding via the lateral intercellular space, or so-called paracellular pathway. By determining an osmotic driving force for water absorption, active sodium transport drives paracellular calcium mobility either by producing a concentration gradient for calcium diffusion or by convection/solvent drag. Otherwise, calcium absorption in distal tubules is completely transcellular and

energetically active. PTH promotes active calcium absorption via a cellular transport mechanism (50,51). Especially, in proximal tubules and thick ascending limbs calcium and sodium absorption happen simultaneously, where elevation of sodium movement is followed by increased calcium transport. Otherwise, reduced sodium transport, for example, that attend the effect of loop diuretics such as bumetanide or furosemide, are followed by reduced rates of calcium absorption. This effect is not observed in distal tubules, where calcium and sodium movement are inversely related. At this level, reduced sodium absorption after thiazide diuretic administration, is associated with elevated calcium absorption. Therefore, in cortical thick ascending limbs, early and late distal convoluted tubule active calcium transport follows a cellular pathway, mediated by PTH. The PTH1R, a member of the G protein–coupled transmembrane receptor family B (47), is abundantly expressed throughout the renal tubule (52). Calcium reabsorption happens both transcellularly through receptor potential cation channels, TRVP5 (53) on the luminal membrane of the distal nephron, and paracellularly through claudins, a family of tight-junction membrane proteins, the proximal tubule, and the cortical thick ascending limb (CTAL) (54). PTH mediates TRVP5-mediated calcium influx distinctly by an indirect mechanism and involves the calcium-sensing protein calmodulin (55). Calcium influx through TRVP5 determines rapid channel inactivation, which avoids excessive calcium absorption. PTH activates the cAMP–PKA pathway, that is necessary and adequate to promote calcium channel entry, at least in late distal convoluted tubules (56). Net calcium absorption requires that after calcium entrance in the distal tubule it must go through basolateral cell membranes. The plasma membrane calcium ATPase (PMCA, ATP2B1) and the Na/Ca exchanger (NCX1, SLC8A1) have been involved in this mechanism (57).

1.4.3 PTH and Vitamin D

The biologically active form of vitamin D is 1,25(OH)2D3 (calcitriol). Stated briefly, it is produced after the sequential hydroxylation of cholecalciferol in the liver by the 25-vitamin D hydroxylase (CYP2R1) and then 1α -hydroxylation by 25-hydroxyvitamin D3 1α -hydroxylase (CYP27B1) in the kidney. The last step occurs in

proximal tubules and is highly mediated by PTH. 1,25(OH)2D3 is degraded to 24,25(OH)2D3, an inactive metabolite, via side-chain oxidation regulated by 1,25(OH)2D3 -24-hydroxylase (CYP24A1), and this process is also mediated by PTH action, in both proximal and distal tubule cells (58). 1,25(OH)2D3 concentrations are regulated not only by PTH action but also by dietary calcium and phosphate, FGF23 and by 1,25(OH)2D3, which inhibits the 1α –hydroxylase (59), induces CYP24, and also suppresses PTH transcription (60), in a tightly regulated negative feedback circuit. Additionally to its stimulatory impact on the 1α -hydroxylase, PTH down-regulates vitamin D receptor (VDR) expression (61). PTH-mediated down-regulation of renal VDR may block suppression of the 1α –hydroxylase by 1,25(OH)2D3 and induction of the 24-hydroxylase, respectively, therefore increasing serum 1,25(OH)2D3 concentrations (62).

1.5 Hypocalcemia

Hypocalcemia is commonly observed in acute inpatient and intensive care unit (ICU) settings and in minor part in the outpatient clinic. In about 15% and 85% of hospitalized and critically ill patients, respectively, hypocalcemia is estimated to happen. True hypocalcemia, defined by a low circulation ionized serum calcium level, is far less frequent than is a low serum total calcium (Ca++) due to alterations in serum albumin or acid–base status (63). For practical purposes, serum total calcium can be used to evaluate hypocalcemia in outpatients setting meanwhile serum albumin is concomitantly determined to calculate albumin-adjusted serum total calcium by the formula: Corrected calcium (mg/dL) = measured total Ca (mg/dL) + 0.8 (4.0 - serum albumin [g/dL]), where 4.0 represents the average albumin level.

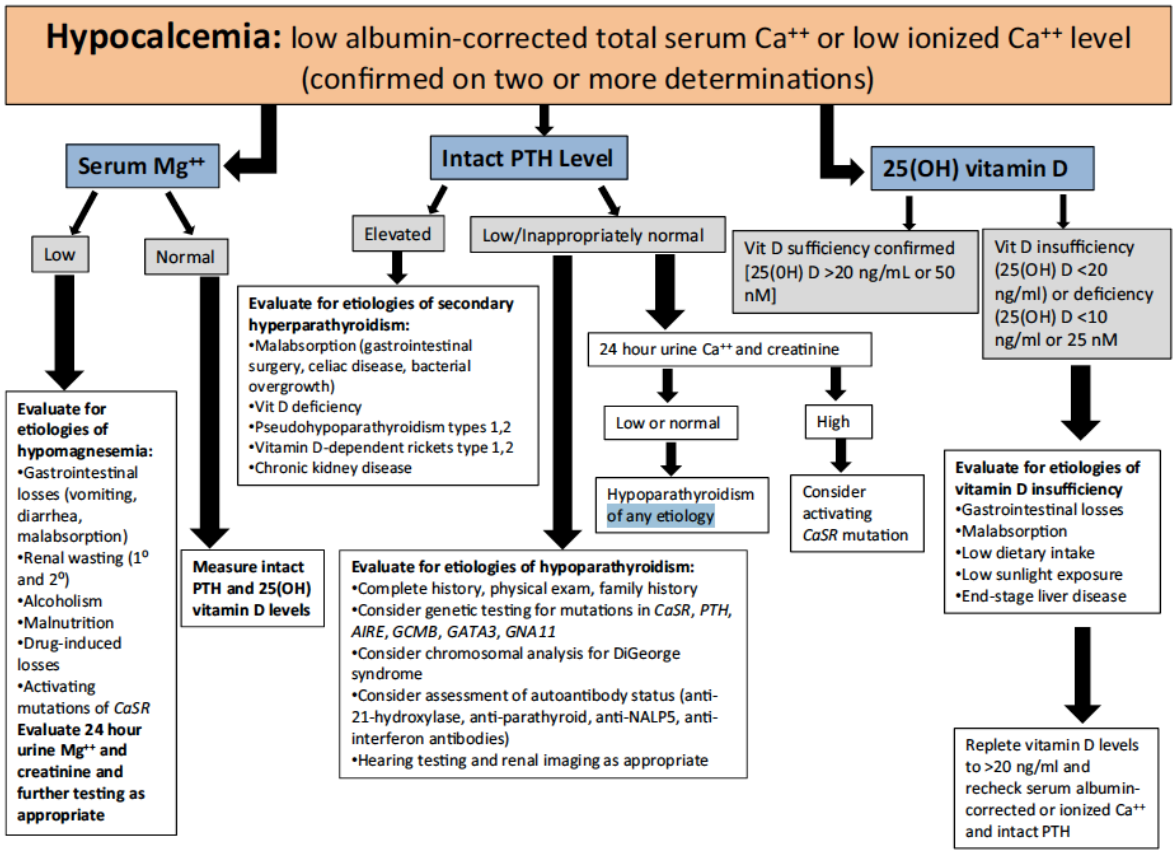
In ICU patients, disorders in serum magnesium (Mg++), phosphate, citrate (due to multiple transfusions), and pH frequently determine a complicate picture of hypocalcemia. This is why measuring the ionized Ca++ is strongly recommended in acutely ill patients. The determination of ionized Ca++ is often fewer available in

outpatient clinical laboratories and is less important due to straightforward circumstances. In postoperative patients, reduced diet intake and hemodilution after infusion of intravenous fluids may clarify transient hypocalcemia, which is frequently mild and asymptomatic. If serum albumin is reduced in hospitalized subjects and corrected calcium level remain in the normal range, no specific treatment or detailed workup is usually needed (64). In subjects who have undergone recent laryngeal surgery or neck surgery for thyroid or parathyroid disease, postoperative hypocalcemia is observed quite differently. All of these procedures may be followed by acute hypocalcemia of either a transient or permanent nature. If hypocalcemia determine clinical signs or symptoms and it is impressive from the laboratory standpoint, further biochemical and hormonal examination must be undertaken. The "hungry bone syndrome" must also be counted in these conditions because chronic hyperparathyroidism or thyrotoxicosis (reasons for neck surgery) may determine postoperative hypocalcemia (65). Hungry bone syndrome will resolve after restoration of bone Ca++ stores but can demand days to weeks of rather significant calcium supplementation (66). This condition could be taken into consideration in presence of an appropriately elevated PTH level and high alkaline phosphatase activity.

1.5.1 Diagnosis and causes of hypocalcemia

For the assessment of a low adjusted for albumin serum Ca++ or serum ionized Ca++ confirmed twice, the physician must evaluate the patient clinically. Therefore, serum Mg++ , creatinine, intact PTH, and 25(OH)D3 levels should be measured (Figure 3) (67). As one puts together the biochemical profile, clinical observations and historical details are very useful to reach an accurate diagnosis efficiently.

Figure 3. Clinical Flow chart for hypocalcemic subjects evaluation (67).



Some features are useful in establishing the accurate diagnosis responsible for hypocalcemia:

The age of onset of the disorder. When hypocalcemia presents in the earlier part of life more likely a genetic disorder will be identified. Several reports reported severe symptoms with significant complications of hypocalcemia and hypomagnesemia for newborns with activating mutations in the extracellular CaSR (68). These complications may be represented by seizures, failure to thrive, growth retardation, or others (69). Hypocalcemic hypoparathyroidism due to autoimmune polyglandular syndrome (APS1) may be diagnosed in childhood (70). In the first years of life, subjects with mutations in AIRE (autoimmune regulator) and APS1 usually present mucocutaneous candidiasis. Different genetic forms of hypoparathyroidism, due to mutations in the genes encoding the transcription factors GCMB (glial cell missing b) (71) or GATA3 (GATA binding protein 3) (72) or in genes encoding PTH (73) or the G-protein alpha subunit GNA11 (G-protein alpha subunit 11) (74) may be diagnosed

at any age, related with the severity of the hypocalcemia.

Presence of renal anomalies and hearing loss. These are important observations in subjects with GATA3 deficiency. Loss-of-function mutations in this transcription factor determine renal anatomic abnormalities and varying degrees of hearing impairment after damaged otic vesicle development (75).

Mental retardation: This aspect may be correlated with the Sanjad–Sakati and Kenny–Caffey syndromes, extremely rare causes of hypoparathyroidism with cardiovascular and facial abnormalities. Mental retardation is also observed in pseudohypoparathyroidism type 1a.

Syndromic features. Expressions of the DiGeorge, velocardiofacial or 22q11.2 deletion syndrome may be represented by cleft palate, abnormal facies, hearing loss, feeding problems, developmental delay, immunodeficiency, the tetralogy of Fallot, persistent truncus arteriosus, ventricular septal defect, and hypocalcemic hypoparathyroidism (76). Pseudohypoparathyroidism type 1a presents multiple phenotypic features (shortened metacarpal bones, obesity, ectopic ossifications, short stature, and mental retardation), despite it is not frequently confused with hypoparathyroidism due to the increased PTH level. Alopecia, delayed growth, and rickets are observed in vitamin D resistance because of mutations in the vitamin D receptor. The Kenny–Caffey syndrome presents short stature, thickening of bone cortices, and basal ganglia calcifications. Mitochondrial DNA defects determining syndromes that include hypoparathyroidism are represented by (77): The Kearns–Sayre syndrome; MELAS (mitochondrial encephalopathy, lactic acidosis, stroke-like episodes); The MTPDS (mitochondrial trifunctional protein deficiency syndrome). However, among the inherited causes of hypoparathyroidism, which are infrequent, these disorders are exceedingly rare.

Conditions dominated by Mg++ deficiency: subjects hospitalized for non-endocrinological diseases often have low serum Mg++ values (e.g., chronic diarrhea, malnutrition, acute pancreatitis, acute and chronic alcoholism, etc.) (78). Due to the

impairment of PTH secretion by chronic Mg++ depletion, serum Ca++ is often reduced.

1.5.2 Clinical evaluation

The presentation of hypocalcemic patient may be ranged from a completely asymptomatic one to dramatic symptomatic and even life-threatening pictures. Neuromuscular and neurologic symptoms are frequently the serious ones the clinician detects. They may feature generalized seizures, loss of consciousness, altered mental status, paresthesias, muscle cramps and tetany. When laryngeal and respiratory muscles are affected, laryngospasm, stridor, and wheezing may be observed and must be treated immediately. Chronic hypocalcemic cardiomyopathy determining congestive heart failure may show volume overload, pulmonary edema, arrhythmias, and profound dyspnea (78). Any cause of acute hypocalcemia may determine central nervous, neuromuscular, neurologic, or respiratory system complaints. Severe chronic hypocalcemia of any etiology may determine cardiac failure. Postsurgical hypoparathyroidism, due to its onset postoperatively, frequently determines complaints that become correlated with the impaired serum Ca++ levels. Physical examination may detect positive Chvostek's or Trousseau's signs indicative of neuromuscular irritability. These signs are quite specific for tetany because of low Ca++ or Mg++ concentrations. Any of the classic observations of pseudohypoparathyroidism type 1a (short stature, obesity, brachydactyly, etc.) support that diagnosis and not one of true hypoparathyroidism. For DiGeorge or velocardiofacial syndrome fluorescence in situ hybridization (FISH) and other genetic analyses could be done. Signs or other symptoms of the APS1 should be sought in subjects with unexplained hypoparathyroidism. Subjects with syndromic manifestations benefit from the diagnostic experience of a geneticist who can also direct specific testing.

1.6 Hypoparathyroidism

1.6.1 Introduction

Hypoparathyroidism is a rare disorder defined by low serum calcium and low or inappropriately low-normal serum PTH. This disorder may be acquired or inherited. The acquired form results after the inadvertent removal of, or damage to, the parathyroid glands or their blood vessels during neck surgery for thyroid disease or parathyroid disease in approximately 75% of cases. In the other cases, the frequent cause in adults is the autoimmune disease, either interesting only the parathyroid glands, or multiple other endocrine glands. Numerous rare infiltrative disorders, metastatic disease, iron or copper overload, ionizing radiation exposure, or rare genetic disorders explains the residual cases (79).

1.6.2 Epidemiology

Hypoparathyroidism clearly complies with the criterion of a rare or orphan disease in the United States with less than 200,000 in the population. In the United States, its prevalence has been estimated to be 37/100,000 (80,81). In Italy, the incidence range from 5.3 to 27 per 100,000 (82,83). In Denmark, the incidence is around 22/100,000 (84,85) and it is less frequent in other countries, such as Norway, where the prevalence is estimated to 9.4/100,000 (86). Some authors reported an incidence of postsurgical temporary hypoparathyroidism of 25.4–83% (87) and permanent hypoparathyroidism ranged from 0.12 to 4.6% of cases (88). Autoimmune hypoparathyroidism is considered the second most frequent cause of hypoparathyroidism in adults. Autoimmune isolated hypoparathyroidism may happen rarely with a low remission rate of 3.8% (89). Autoimmune hypoparathyroidism may also be found in association with other autoimmune endocrine disorders as part of APS-1 (89). The worldwide incidence of APS-1 is estimated to be 1/1,000,000 person-years, but the incidence is more frequent in three genetically distinct populations: 1:25,000 in Finns, 1:14,500 in Sardinians, and 1:9000 in Iranian Jews (90). Despite the variability within and between countries, hypoparathyroidism remains a rare disorder of calcium homeostasis.

1.6.3 Surgical hypoparathyroidism

Surgical hypoparathyroidism is defined by an inadequate production of PTH occurring after surgery. Surgical hypoparathyroidism can be divided in two main categories: following total or subtotal thyroidectomy; and following parathyroid surgery (after the removal of a single or several parathyroid adenoma) in either PHPT or SHPT.

The significance of occurring after thyroid surgery is more important epidemiologically due to the higher number of thyroid than parathyroid surgery that are performed. Independently of the kind of neck surgery, this form of hypoparathyroidism is frequently evident within a few hours after the operation, but also after 1 or 2 days postoperatively. Postsurgical hypoparathyroidism can be transient (ranged from a few weeks to 6 months) or permanent (after 6 months).

Hypoparathyroidism after thyroid surgery. Throughout thyroid surgery, parathyroid glands may be devascularized by ligation of thyroid arteries near to the origin of the parathyroid arteries, thermally damaged by accidental electrocoagulation, and, often, by inadvertent removal, or damage. The parathyroid glands required always be identified at the time of thyroidectomy. To maintain their blood supply, it is crucial to ligate thyroid arteries as distal to the origin of parathyroid vessels as possible. When parathyroid glands are accidentally damaged various grades of parathyroid insufficiency may be observed (91). The rate of surgical hypoparathyroidism greatly depends upon surgical technique but also on the complexity and duration of the operation: extensive thyroid surgery, radical neck lymph node dissections are factors influencing the risk of hypoparathyroidism. Another aspect playing an important role is the repeated surgery, often associated with a higher hypoparathyroidism risk (92). Some pre-existing clinical situations also may play a significant role in the development of postoperative symptomatic hypocalcemia, such as: hypovitaminosis D, magnesium depletion or high bone turnover rate (due to preoperative severe hyperthyroidism).

Hypoparathyroidism after parathyroid surgery. In parathyroid surgery, beyond the

experience of the surgeon more important is the anatomic and physiologic integrity of the remaining parathyroid glands. Usually, the glands left behind during parathyroid surgery are not functional due to the overactive parathyroid tissue that physiologically suppressed them. When PHPT or SHPT is severe, the remaining parathyroid tissue might demand a short period of time before it recovers. Hypoparathyroidism is a significant but relatively less frequent complication of parathyroid surgery (93).

Clinical aspects. The clinical features of surgical hypoparathyroidism are characteristic, especially when its onset happens during the first postoperative day when the reduction in calcium levels may be severe and immediate treatment is mandatory. If the decrease in serum calcium is mild, hypocalcemia may be asymptomatic. Acute hypocalcemia is followed by symptoms of neuromuscular irritability and neurological ones and sometimes also electrocardiographic alterations. Firstly, numbness and tingling in the fingertips and circumoral region are observed, followed by paresthesias in the extremities. If serum calcium levels decline acutely, signs of tetany such as carpal or pedal spasm will appear (the Trousseau sign is peculiar). Another typical manifestation reported is the Chvostek sign, consisting of a contraction of facial muscles after light percussion on the facial nerve near its outlet close to the external auditory meatus. Uncommon symptoms that may cause extreme concern for patients are broncho- and laryngospasm. Cardiologic alterations determined by hypocalcemia are frequently expressed by prolonged QT interval on electrocardiogram and rarely arrhythmias or congestive heart failure (94). If serum calcium levels reduction occurs gradually 3 days or more after surgery, hypocalcemia can become clinically manifest several days after the operation (95). The perioperative dosage of albumin-corrected serum calcium or ionized calcium represent a reliable way to exclude or confirm postsurgical hypoparathyroidism. Intact PTH and phosphate measurement can further establish parathyroid gland competency after surgery (96). Low PTH and high phosphate concentrations are evidences of a hypoparathyroidism state, while normal concentrations of PTH and phosphate can be reassuring. Serum magnesium levels may also help to sort out the presence of frank hypoparathyroidism

(97). During hypomagnesemia, PTH secretion is suppressed along with a peripheral PTH resistance, thus increasing and prolonging hypocalcemic symptoms. This effect of magnesium is reversible with magnesium replacement.

Prediction of surgical hypoparathyroidism. The onset of surgical hypoparathyroidism is one of the main events limiting early hospital discharge for subjects after thyroid or parathyroid surgery, especially when it happens on the first or second postoperative day. Considering the adoption of minimally invasive techniques (98) and the use of local or loco-regional anesthesia (99), it would be highly favourable to understand the factors that can predict surgical hypoparathyroidism, and to implement adequate interventions to prevent this outcome. Measuring PTH serum levels during surgery or immediately thereafter appeared to be the most reliable and the earliest predictive element for the onset of surgical hypoparathyroidism (95).

Determination of circulating PTH after surgery in association with serum calcium levels at 6 or 12 hours, is frequently accept for assessing the likelihood of postsurgical hypoparathyroidism (100). Accurate surgical technique represents the best prevention. Despite all precautions, postoperative hypoparathyroidism may occur so an accurate work-up of the patients' metabolic state prior to surgery is advisable, especially vitamin D and magnesium state. In subjects with a higher risk of hypoparathyroidism, such as those who undergo complex operations for severe neoplastic disease, repeated surgery, or suffering from Graves' disease, it may be beneficial to initiate treatment with calcitriol prior to or quickly after surgery (97).

1.6.4 Complications

1.6.4.1 Skeletal impairment

Bone turnover markers. Serum markers of bone formation such as procollagen type 1 amino-terminal propeptide (P1NP), osteocalcin (OC), or bone-specific alkaline phosphatase (BAP) and markers of bone resorption such as tartrate-resistant acid phosphatase 5b (TRAP-5b) and serum C-telopeptide (CTX) are generally in the lower half range in subjects affected by hypoparathyroidism (101–103). In association of

reduced marker values, Rubin et al. reported also reduced numbers of circulating osteogenic precursor cells (104) and increased circulating sclerostin levels (105).

Bone mineral density. The chronically low bone turnover state in hypoparathyroidism determines higher BMD compared to age- and sex-matched controls (106). Expected postmenopausal bone loss from estrogen deficiency seemed to be lower compared to women without hypoparathyroidism (107). Trabecular volumetric BMD (vBMD), cortical vBMD and cortical thickness measured by peripheral quantitative computed tomography are all greater in hypoparathyroidism than controls (108). Higher cortical vBMD is also highlighted by high-resolution peripheral quantitative computed tomography (109).

Histomorphometry aspects. More revealing are the results from studies by dynamic histomorphometry in which all cancellous, endocortical, and intracortical bone sites are impaired (110). The impact of the lack of PTH on cancellous and cortical bone mass, which was noted initially by non-invasive imaging and by two-dimensional (2D) histomorphometry, was confirmed by the three dimensional (3D) analytical capability of microcomputed tomography (μ CT) (111).

Fracture risk. Information on fracture risk in hypoparathyroidism are sparse. By finite element analysis, mechanical strength appears to be normal in hypoparathyroidism and an increase or decrease in fracture risk was not observed (112). The skeletal impairments that have been seen, represent a reason to be concerned about bone fragility in hypoparathyroidism (110), but further data are necessary to clear this point.

1.6.4.2 Other complications

Renal. The lack of PTH's calcium-conserving properties determines an increase in the glomerular filtered calcium (65). Although renal function can be preserved, the kidneys are a major target organ for complications. The replacement therapy for hypoparathyroidism can lead to hypercalciuria, due to PTH insufficiency impairs renal calcium reabsorption (113). Hypercalciuria may be followed by nephrolithiasis, nephrocalcinosis, and renal insufficiency (114). The rates of asymptomatic

nephrolithiasis and nephrocalcinosis in patients with hypoparathyroidism was reported from 8% to 40% (114,115). Subjects with nonsurgical hypoparathyroidism seemed to have more renal dysfunction compared to the postsurgical form, probably because they had lived with this condition for a longer time (79).

Cataracts. Cataracts has long been related with both postsurgical (55%) (116) and idiopathic hypoparathyroidism (41–51%) (117). The cataracts seem to be posterior and begin in the periphery of the lens.

Skin. Several dermatological manifestations include dry, scaly skin, brittle nails, coarse and thin hair, and, uncommonly, a pustular psoriasis (116). Candidiasis is a specific manifestation observed in patients with the autoimmune form of hypoparathyroidism.

Basal Ganglia Calcification. Basal ganglia calcifications are a well-known manifestation of chronic hypoparathyroidism. Longer duration of disease appears to be associated with basal ganglia calcification, or even more diffuse brain calcification (118). While this manifestation is usually asymptomatic, sometimes may be associated with cognitive dysfunction (118), or with organic mood disorder (119). Reported rates of basal ganglia calcification differ, from 12% to 36% of patients with CaSR mutations (120); and up to 52% in postsurgical hypothyroidism cases. Serum phosphorus levels have been found to be higher in subjects with basal ganglia calcification in comparison to those without.

1.6.5 Quality of life

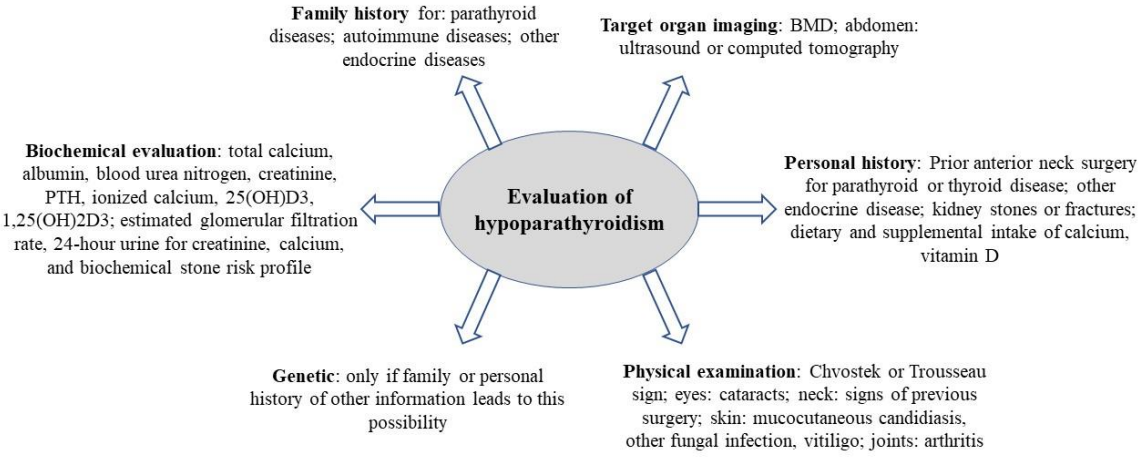
A uniform viewing across the literature is that quality of life (QOL) in subjects with hypoparathyroidism is reduced independently if serum calcium is controlled by conventional therapy (121,122). One of the most prototypical features complain spontaneously by patients with hypoparathyroidism is the "brain fog". The patient's perception is that thinking has been clouded normally and pervasively (123). Most QOL studies have utilized the generic 36-Item Short Form Health Survey (SF-36), a validated measure of health-related QOL (79,121) that resulted reduced in

hypoparathyroidism compared to controls. The SF-36 scale evaluates both physical and mental domains, virtually all of which are adversely impaired. Reduced QOL is not as readily perceived by health care professionals, like surgeons, as they are by their patients (124). It should be reported that the SF-36 instrument is a generic questionnaire, and it remains to be investigated how subjects with hypoparathyroidism would score a disease-specific questionnaire.

1.6.6 Evaluation and management

Evaluation. Current recommendations for the evaluation of a patient with hypoparathyroidism are summarized in Figure 4 .

Figure 4. Evaluation of hypoparathyroidism (79)

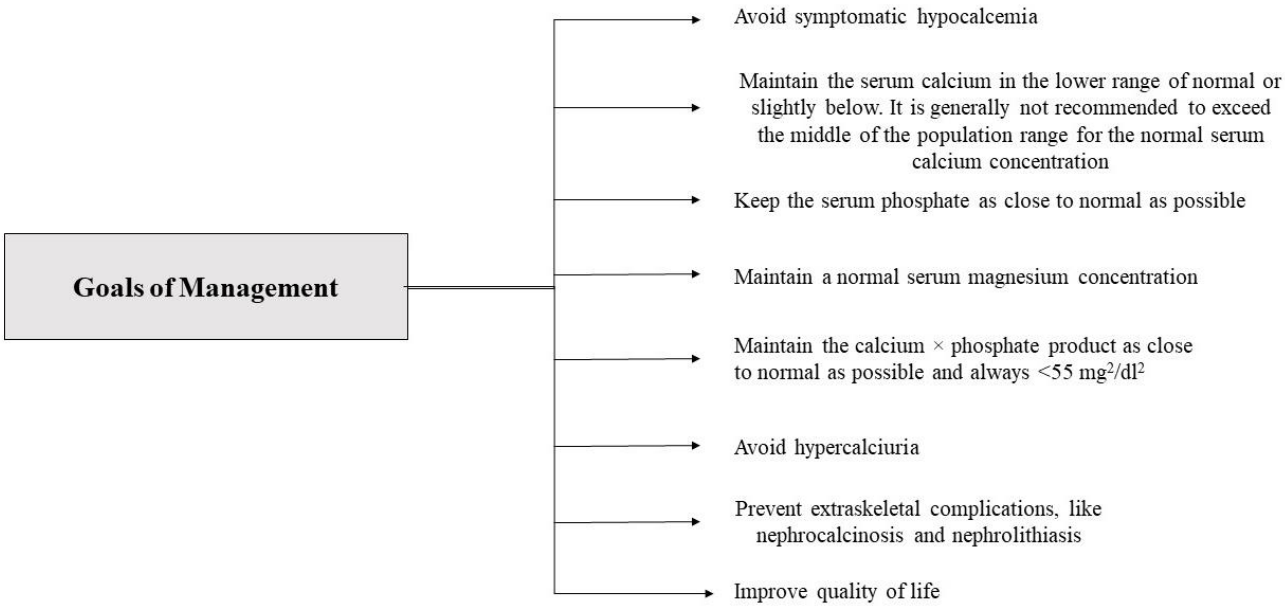


Acute management. Subjects with hypoparathyroidism can become acutely hypocalcemic either immediately after of neck surgery or when chronic replacement regimen has been perturbed by gastrointestinal illness or other factors. If symptoms are present, a rapid action is needed (125), using an intravenous infusion of 1 to 2 ampules of 10% calcium gluconate (93 mg of elemental calcium/10 mL) in 50 mL of 5% dextrose over 15 to 30 minutes. After that, a second step is a slower and prolonged infusion of calcium gluconate, 0.5–1.5 mg/ kg body weight/hour, over an 8- to 10-hour

period. During both phases, continuous electrocardiogram monitoring is recommended, and calcium levels should be monitored every 4 to 6 hours.

Chronic management. The major goals for chronic management are to avoid symptomatic hypocalcemia for patients as well as to ameliorate/prevent the complications of the disease (125) (Figure 5). Conventional therapy with calcium and vitamin D generally cannot lessen the complications of the disease and sometimes may increase the risk due to the high doses that are often necessary. The natural history of hypoparathyroidism has not been prospectively ascertained and cross-sectional studies are confused by the treatment that could not be discontinued.

Figure 5. Goals of Management (79)



Conventional therapy. The goal of the conventional therapy using supplements of calcium and vitamin D is to keep the serum calcium within the low normal reference range, namely 8.0 to 8.5 mg/dL or even somewhat below the lower limit for a normal population meanwhile the patient is asymptomatic (125). Usually, patients will require 1 to 2 g of supplemental calcium given in splitted doses of 500 mg at a time. Calcium carbonate is typically preferred due to the fact that 40% of this calcium salt is elemental calcium by molecular weight, making it a more efficient source. Not always calcium

carbonate is well tolerated, sometimes it complains bloating and constipation. Alternatively, calcium citrate can be used, and it is more advantageous because it does not require a source of acid and is not typically associated with constipation (8). In hypoparathyroidism, active vitamin D formation is impaired in the kidney due to the lack of PTH and hyperphosphatemia and it is why the active form of vitamin D, calcitriol, or an active analogue, 1-alpha hydroxycholecalciferol, is required. The active vitamin D has a biological half-life of 4 to 6 hours and daily or twice-daily administration is needed. The average daily dose of calcitriol is about 0.5 to 1.0 mcg or 1.0 to 2.0 mcg of the 1-alpha analogue. In patients with hypoparathyroidism it seems reasonable to maintain levels of 25-hydroxyvitamin D at > 20 ng/mL (50 nmol/L) by using cholecalciferol (vitamin D3) or ergocalciferol (vitamin D2) (126).

Parathyroid hormone. Conventional therapy with calcium and active vitamin D does not solve the basic problem in hypoparathyroidism, namely the hormone is lacking. Some authors demonstrated that the use of PTH(1-34) improved control of the serum calcium with smaller amounts of supplemental calcium and vitamin D (127,128). The use of the native human hormone, recombinant human PTH(1-84) (rhPTH(1-84)) ushered in the official therapeutic era of full hormone replacement therapy. The pivotal study with rhPTH(1-84) (REPLACE) demonstrated an adequate maintenance of the serum calcium with 50% or greater reduction in starting oral calcium and active vitamin D doses (129). This study was followed by REPEAT, that confirmed and repeated these findings (130). Long term therapy with rhPTH(1-84) determined a reduction in urinary calcium excretion (131) and an improvement in quality of life (132).

1.7 Primary hyperparathyroidism

Primary hyperparathyroidism is characterized by hyperactivity of one or more parathyroid glands with a consequent increase in serum calcium and concomitant high or inappropriate circulating levels of PTH (133). Historically, PHPT was characterized

best by skeletal and kidney involvement. From the 1970s, this clinical phenotype changed rather dramatically with the increase in biochemical screening tests and diagnosis of PHPT in presence of mild hypercalcemia and lack of any specific symptomatology (134). The greatest part of patients with PHPT have a single, benign adenoma and only a smaller percentage (about 15% to 20%) have multigland disorder, including multiple adenomas and hyperplasia. Multigland disease is more frequent in familial syndromes such as multiple endocrine neoplasia (MEN) 1 or 2 (135). Parathyroid carcinoma is rarely observed, occurring in fewer than 1% of subjects with PHPT (133) and may be associated with gene mutations. These genes include MEN1, CaSR, HRPT2, RET (familial forms), and PRAD1/cyclin1 (sporadic tumors) (136).

1.7.1 Diagnosis

PHPT is characterized by hypercalcemia and concentrations of PTH that are inappropriately elevate for the hypercalcemic state. Usually, the PTH concentration is frankly elevated, but it can also be within the normal range. In both situations, within normal or high levels of PTH are clearly inappropriate when the serum calcium is elevated. The differential diagnosis of PTH-dependent hypercalcemia contains the concomitant therapy with thiazide diuretics or lithium (137,138). Other conditions that need to be excluded are familial hypocalciuric hypercalcemia (FHH), vitamin D deficiency, bisphosphonates, and renal failure including tertiary hyperparathyroidism (139). Hypercalcemia associated with very low or undetectable PTH levels may be found in malignant diseases, where PTHrP determines often elevation of serum calcium (140). PTH assays have evolved significantly and various generations of PTH assays are used. The older ones, called "intact" PTH or second-generation PTH assays cross-react with an N-terminal truncated PTH fragment called (7–84) PTH or non-(1–84) PTH (141). The more recent assay called third generation do not detect the non-(1–84) PTH but a post-translational form called amino PTH (142).

1.7.2 Epidemiology

PHPT predominates among postmenopausal women, with a female/male ratio of 3 to

4:1. The prevalence varies by country and race. In the United States, PHPT has a prevalence of 0.86% (143), with a racial predilection that appears to favour blacks (144). In the last years, a progressive rise in incidence and prevalence rates has been reported probably resulted from the greater recognition and diagnostic pursuit of mild forms of PHPT and the likelihood that biochemical screening is even more widespread than before (139). In countries such as China or Latin America, where serum calcium has become routinely evaluated, the incidence of PHPT has increased (145,146).

Epidemiology in subjects with Head or Neck Radiation Exposure. Ionizing radiation is a known risk factor for sporadic PHPT development attributable to parathyroid adenomas (147). Use of therapeutic head/neck radiation for benign conditions such as acne has been correlated with parathyroid adenomas surgical treated years later (148). Survivors from the Hiroshima atomic bomb as well had an increased risk of PHPT (149).

1.7.3 Sex and age distribution

PHPT affects postmenopausal women more frequently than men, but without difference before menopause. Indeed, in men and women <45 years of age the incidence rate is mainly the same, whereas after menopause, the ratio of incidence in women to men is 2:1. Increasing age is as well related with grater incidence rates in both men and women, with a higher influence in women. The highest incidence rate of PHPT is observed in women aged between 65 and 74 years, with an annual incidence of 99/100,000 person-years in comparison with a rate of 17.2/100,000 person-years in men of the same age (150).

1.7.4 Mortality

Conflicting information is available regarding the risk of death in subjects affected by PHPT. In Europe, data on mortality are based largely on subjects with more severe forms of PHPT, treated surgically who showed a higher risk of death (151). Some studies reported a normalization of the increased mortality risk 5 years after parathyroidectomy in subjects with milder PHPT but others suggested a persistently

elevated risk of death several years after surgery (148). In Europe, patients with mild to moderate PHPT seemed also to have an increased risk of death (152). Untreated PHPT appears to be associated with increased cardiovascular mortality (153,154)

1.8 Clinical aspects of primary hyperparathyroidism

The presentation of PHPT has followed a timeline with the symptomatic form described first, then the asymptomatic PHPT, and, most recently, the normocalcemic hyperparathyroidism (NHPT). Even if this historical perspective is valuable, it is relevant to admit that all these forms of PHPT exist concomitantly in the world today and probably have always coexisted. The preponderance of one form over another depends on numerous factors that tend to differ by country. In states where biochemical screening is not regularly done, is easily to observe most frequently the symptomatic PHPT form. India might be one of the best examples of this form of PHPT. In Western Europe, North America, and many other countries where biochemical screening is routine, asymptomatic PHPT has become the predominant form of presentation (139). If PTH is routinely detected during screening for low BMD, even in normocalcemic subjects, NHPT will emerge as an entity (139).

1.8.1 Bone disease

The classic and severe bone disease of PHPT is osteitis fibrosa cystica, determining bone pain, deformities, and pathologic fractures. Increased bone resorption because of the elevated levels of PTH is associated with numerous typical radiologic signs. Sub-periosteal bone resorption of the distal phalanges is the most specific radiologic feature. It is well observed on the radial side of the middle phalanges. Radiologic alterations may be present in the skull, recognized as "salt-and pepper" pattern. Local destructive lesions, bone cysts, and "brown tumors" in the long bones and pelvis represent other skeletal manifestations. All these features are associated with pronounced reduction in BMD at all sites. Bone density (DXA) tends to indicate preferential BMD loss at cortical sites (the distal one-third site of the forearm) whereas sites rich in cancellous

bone such as the lumbar spine are preserved (155). Latest non-invasive skeletal imaging technologies, however, offer new overviews. Trabecular bone score (TBS), which offers an indirect evaluation of trabecular microstructure using the DXA image and predicts fracture despite BMD, shows trabecular impairment independently of preserved BMD in PHPT (112,156,157). Data resulted from high-resolution peripheral quantitative computed tomography (HRpQCT), a research tool that non-invasively and directly evaluates skeletal microstructure, have shown trabecular impairment at the radius and tibia (156,158,159). These findings help to explain the increased risk of VFx described in PHPT prior to the availability of these technologies (160,161). The Guidelines for the Management of Asymptomatic PHPT (2014) took these findings into consideration by recommending screening for VFx and surgical indication if present (162). After parathyroidectomy, fracture risk seems to diminish. In patients non treated surgically, antiresorptive drugs have been demonstrated to increase BMD, but there are no data available on whether these drugs decrease fracture risk in PHPT (163).

1.8.2 Renal disease

The most frequent renal effects of PHPT are hypercalciuria and nephrolithiasis. This classical sign is clinically easy to recognize, has decreased during recent decades, but remains an undisputed indication for parathyroidectomy (164). Most series report a rate of kidney stones about 15–20% of PHPT, with a higher frequency detected by ultrasound screening in subjects with asymptomatic PHPT (165). An even higher frequency of occult nephrolithiasis (36%) was documented from an Italian cohort of asymptomatic PHPT (166). Besides nephrolithiasis, the kidneys may be affected in other ways, such as deposition of calcium phosphate crystals throughout the renal parenchyma, known as nephrocalcinosis. Nephrocalcinosis may or may not be associated with frank stones and/or a decrease in creatinine clearance (167).

1.8.3 Other clinical manifestations

Besides the skeleton and the kidneys, PHPT has the potential to involve other organ systems.

Neurological and psychiatric disease. Weakness and fatigue have been associated with a specific neuromuscular syndrome, characterized histologically by atrophy of type II muscle fibers (168). Some studies report, that even mild forms of PHPT are associated with depression, anxiety, irritability, decreased quality of life (QOL), sleep disturbances and cognitive impairments (169,170). After parathyroidectomy, an improvement has been observed (171–173).

Rheumatological and gastrointestinal features. Typically, PHPT was uncommonly associated with hyperuricemia, gout and calcium pyrophosphate crystal deposition (174). Pseudogout, or synovitis determined by calcium pyrophosphate crystal deposition, has been documented after parathyroidectomy, even if the mechanism is not clear (175). Pancreatitis and peptic ulcers are not predominantly seen in patients affected by PHPT due to mild hypercalcemia, meanwhile constipation is more frequent associated with PHPT (176–178).

Cardiovascular disease. Despite the increasing evidence that PHPT may support a higher cardiovascular morbidity and mortality, the pathogenic mechanisms are still not completely unequivocal. Numerous disfunctions have been associated with PHPT, including hypertension, premature atherosclerosis, valve calcification, left ventricular hypertrophy, and arrhythmias (179) (180). Some studies have documented increased vascular stiffness, occasionally associated with PTH concentrations, in mild forms of PHPT, but its reversibility after parathyroidectomy remains variable (181,182).

1.8.4 Quality of life

PHPT appears to be characterized by impaired QOL, measured by SF-36, but surgery provide conflicting results regarding the improvement of QOL (169,183). The last International Workshop, therefore, did not add reduced QOL as a criteria for parathyroidectomy but identified the need for more data regarding this feature (162). Recently, has been published the largest and long-term trial indicating an improvement in some of the mental domains of SF-36 after parathyroidectomy but with uncertain clinical significance compared with controls (184).

1.9 Treatment of hyperparathyroidism

1.9.1 Parathyroid surgery

Parathyroidectomy remains the only option to cure PHPT (185). While surgery is recommended in all patients with classical symptoms of PHPT unless medical contraindications, the indications for parathyroidectomy in asymptomatic forms of the disease have been addressed by the last guidelines (Table 1) (162). Even if evidence-based recommendations are lacking in NHPT, parathyroidectomy is suggested if subjects become hypercalcemic and present other indications for intervention. Surgery is also considered in NHPT when a disease progression regardless of hypercalcemia is registered (162). Surgical guidelines are more liberal than those of the Fourth International Workshop for the Management of Asymptomatic PHPT (186). Some of them also recommend parathyroidectomy in subjects with cognitive/ psychiatric symptoms, cardiovascular disease (other than hypertension) associated to PHPT (186).

Table 1. Evaluation, monitoring and indications for surgery in asymptomatic primary hyperparathyroidism (162)**.**

Evaluation and monitoring of patients with hyperparathyroidism	**Indications for surgery in asymptomatic primary hyperparathyroidism**
Serum PTH, calcium, phosphate, alkaline phosphatase activity, renal function tests, 25(OH)D3	Age <50 years
24-Hour urine for calcium and creatinine	Serum calcium >1 mg/dL above upper limit of normal
BMD by DXA (lumbar spine, hip, distal one-third radius)	Reduced BMD by DXA to a T-score of <−2.5 at any site (lumbar spine, hip, or distal one-third radius)

Vertebral spine assessment (radiography, CT or VFA by DXA)	Vertebral fracture by X-ray, CT, magnetic resonance or VFA
Stone risk profile (if urinary calcium >400 mg/day)	Creatinine clearance <60 mL/min
Abdominal imaging by radiography, ultrasonography, or CT scan	Kidney stone or nephrocalcinosis by abdominal imaging
Optional: HRpQCT, TBS, Bone turnover markers	Hypercalciuria (>400 mg/day) accompanied by biochemical stone risk profile placing patient at risk of kidney stones

Preoperative localization is used in subjects candidates for minimally invasive parathyroidectomy as well as in disease that is recurrent or persistent after surgery (187–189). These techniques have no role to make the diagnosis; rather, they should guide the surgeon once a diagnosis is made. The most widely used localization modalities are technetium-99m-sestamibi [with or without single photon emission computed tomography (SPECT)] or ultrasound. Negative imaging, frequent in multiglandular PHPT, is not unsubstantial with the diagnosis and does not preclude surgical option (186). Recently, ^{11}C-Methionine PET/CT showed a good performance in localizing hyperfunctioning parathyroid glands in PHPT subjects with inconclusive pre-operative imaging (190–192).

Surgery. Even without localization, an experienced parathyroid surgeon will find the abnormal parathyroid gland(s) in most cases. Focused minimally invasive parathyroidectomy has rapidly became the procedure of choice in subjects in whom preoperative localization has revealed single gland disease (189). This procedure requires the capability to detect intraoperative PTH concentrations. Taking advantage of the short half-life of PTH (3–5 minutes), an intraoperative PTH concentration is drawn shortly following resection (193). When the PTH concentration decrease by 50% and is within the normal range, the adenoma that has been resect is considered to be the only source of abnormal glandular activity, and the procedure is concluded. Potential complications of surgery contain damage to the recurrent laryngeal nerve,

which can lead to hoarseness and decreased voice volume, and permanent hypoparathyroidism in patients who previously undergo neck surgery subtotal parathyroidectomy (for multiglandular disease). After surgery, the subject may experience a short period of transient hypocalcemia. This appears within the first few days postoperatively but can be prevented in most cases by supplementing patients daily with calcium during the first postoperative week. Prolonged postoperative symptomatic hypocalcemia as a consequence of rapid deposition of calcium and phosphate into bone ("hungry bone syndrome") is rare in recent years. Such condition may require parenteral calcium for symptomatic hypocalcemia. After successful surgery, when the subject is cured, serum calcium and PTH concentrations normalize. Available data reported that bone density improves in the first several years after parathyroidectomy (194). The cumulative increase in bone mass at the lumbar spine and femoral neck seems to be sustained for years after surgery. In subjects with osteopenia or osteoporosis an even more important improvement in spine BMD appears after surgery. These findings suggest that patients with these conditions should be routinely referred for surgery, regardless of the severity of their hypercalcemia.

1.9.2 Non-surgical management

Subjects who do not undergo surgery must be monitored and when indication is met during follow-up, parathyroidectomy should be considered (162). Long-term observational data suggest that biochemical indices remain stable for several years in patients followed nonoperatively (195). Moreover, BMD appears to decline at cortical sites after 10 years of follow-up and almost 40% of subjects developed one or more indications for parathyroidectomy over 15 years of observation (194). A set of general medical investigations is recommended during follow-up (162). Serum calcium concentrations should be evaluated once to twice yearly with annual assessment of serum creatinine and annual or biannual bone densitometry at the spine, hip, and distal one-third site of the forearm. Adequate hydration and ambulation are always recommended. Thiazide diuretics and lithium should be avoided if possible, due to their potential hypercalcemic effect. Dietary calcium intake should be moderate, as low

calcium intake could lead to further stimulation of PTH secretion. In postmenopausal women, estrogen therapy could be an option in those desiring hormone replacement in order to treat symptoms of menopause (196). The rationale for estrogen use in PHPT is based on the aware opposition between estrogen and PTH on bone resorption. The prevalence of vitamin D deficiency is more common in subjects with PHPT compare with geographically matched groups (197). Several mechanisms may play a role in vitamin D deficiency development in concomitant PHPT (198). In PHPT, the vitamin D status may impact the clinical expression of the disease, in particular the asymptomatic form (199). Concomitant vitamin D deficiency/insufficiency in subjects with mild PHPT may counteract the impact of PTH on increasing serum and urinary calcium concentrations. Guidelines further recommend replacing vitamin D to levels of 21–30 ng/ml with conservative doses of cholecalciferol (600–1000 IU daily) based on data showing that vitamin D treatment lowers PTH levels (200). Active vitamin D metabolites should not be employed to adjust vitamin D deficiency in PHPT.

Bisphosphonates have been evaluated as a possible medical approach to PHPT. Pamidronate, risedronate, and alendronate are all nitrogen-containing bisphosphonates that have been investigated in PHPT. The major part of the studies assessing bisphosphonate therapy in PHPT used alendronate and this bisphosphonate seemed to effectively reduce bone turnover and increase BMD in subjects with PHPT. Impact on serum calcium concentrations has been inconsistent and serum PTH concentrations have been stable with no statistically significant change. There are very limited data assessing bisphosphonate therapy in men affected by PHPT. Some studies described above included men in their sample but did not compare them with women in terms of effectiveness. In one international study assessing alendronate in PHPT, the male group was examined separately and compared with the postmenopausal women group (201). Bisphosphonate therapy requires further studies regarding its impact on bone strength and fracture risk.

Denosumab demonstrated to be efficient in improving BMD and lowering bone turnover in subjects with PHPT (202). When compared with parathyroidectomy,

denosumab seemed to be equally effective in increasing BMD but also improved TBS (203). These findings suggest that denosumab might be a valid option for patients in which surgery is undesirable or not indicated.

Calcimimetic agents affect the extracellular CaSR function, increasing the affinity of the parathyroid cell calcium receptor for extracellular calcium, determining to increased intracellular calcium, a subsequent decrease in PTH synthesis and secretion, and lastly a fall in the serum calcium level. Cinacalcet normalized and sustained reductions in serum calcium values across a wide range of disease severity (204). Neither BMD nor urinary calcium excretion enhance after treatment. Moreover, there are no available data concerning nephrolithiasis risk reduction. Cinacalcet is approved for use in parathyroid cancer and for the treatment of severe hypercalcemia in subjects with PHPT who are unable to undergo surgery but not currently recommended for general use those who are candidates for operation.

1.10 Normocalcemic hyperparathyroidism

Normocalcemic primary hyperparathyroidism (NHPT) was first described in 2008 and is defined by elevated PTH levels with consistently normal albumin-adjusted and ionized serum calcium concentrations, after exclusion of secondary causes of hyperparathyroidism (205). Several studies aimed to further characterize NHPT have been published but uncertainties still remain about its prevalence and natural history. Up to now, there is not yet a consensus for recommending either medical or surgical management of NHPT.

1.10.1 Epidemiology

Prevalence of NHPT varies in the different cohorts described. In the general population, nonreferral populations, such as The Osteoporotic Fractures in Men (MrOS) study and Dallas Heart Study (DHS), the prevalence reported was 0.4 and 0.6%, respectively (206). Similarly, in a village in Southern Italy, the prevalence of

NHPT detected was 0.44% of an unselected sample of the whole community of adults (207). Moreover, the prevalence of NHPT founded in postmenopausal women evaluated for skeletal health was of 6-8.5% (208,209)

1.10.2 Pathophysiology and diagnosis

Pathophysiology. One of the common hypotheses proposed NHPT as an early, 'subclinical' expression of the more widely described PHPT (210). Other authors proposed that this condition could be linked to a target organ (skeletal or renal) resistance to the actions of PTH due to the inadequate suppression of PTH in response to an oral calcium load reported in NHPT subjects compared to hypercalcemic ones (211).

Diagnosis. The diagnosis of NHPT consists of normal total and ionized serum calcium levels in the presence of consistently elevated PTH concentrations (211), after ruling out all causes of secondary hyperparathyroidism such as vitamin D deficiency and kidney failure. For PTH measurement, both second- and third-generation assays are considered equivalently useful (162). For NHPT diagnosis, serum calcium should be evaluated as either total albumin-adjusted calcium and ionized concentrations. Serum calcium must also be assessed at least twice within a 6-month period in order to discriminate a true normocalcaemic state from a hypercalcaemic one (162). Integral to diagnosis of NHPT is the exclusion of secondary causes of hyperparathyroidism. The most frequent cause is represented by vitamin D deficiency. Usually, high PTH levels should be physiologically suppressible after 3 months of vitamin D supplementation, although particular dose recommendations of vitamin D vary by race (212). To be confident in the diagnosis of NHPT, it would be recommended to consider the 25(OH)D3 level greater than 30 ng/mL. Renal insufficiency is another common cause of secondary hyperparathyroidism. The Kidney Disease Outcomes Quality Initiative (KDOQI) and Kidney Disease Improving Global Outcomes (KDIGO) guidelines report that serum PTH raising tends to initiate with eGFR <60 mL/min/1.73 m2 (213).

The diagnosis of NHPT requires exclusion of some additional conditions such as: malabsorptive disorders (ie celiac disease, cystic fibrosis, biliopancreatic diversion complications), idiopathic hypercalciuria, as well as use of medications that may affect serum PTH and/or calcium concentrations (loop diuretics, lithium, bisphosphonates, denosumab and antiepileptic medications) (214,215).

1.10.3 Clinical aspects of normocalcemic hyperparathyroidism

Bone disease. In patients with NHPT was documented a high prevalence of osteoporosis (46-55%) (216–220). Moreover, the prevalence of VFx in NHPT subjects seems to be lower compared with PHPT (216). Despite the preferential bone loss at the lumbar spine and hip, at the one third distal radius BMD appears to be preserved in NHPT than PHPT (216,217,220). Using HRpQCT both cortical and trabecular microarchitectural abnormalities have been reported in PHPT patients (158), while NHPT subjects showed an impairment in cortical geometric properties with preserved trabecular parameters (221). NHPT population seems to have normal bone turnover when compared with PHPT (206,216,222).

Renal disease. Urinary calcium excretion seems to be equal or even lower in patients with NHPT than PHPT (216,223,224). Nephrolithiasis have been reported in 13–18% of patients with NHPT (216,217,220,222) similarly to PHPT (216,219,220).

Other complications. NHPT may increase cardiovascular risk due to the physiologic impact of PTH and/or calcium on the cardiomyocyte, cardiac conduction system and endothelial cells (225). Recently, was published that NHPT patients had more prominent coronary artery calcification than those with the hypercalcaemic form (226). In literature there are contrasting results in terms of glucose metabolism, lipid profiles and cardiovascular risk scores in NHPT in comparison to PHPT. Some evidences demonstrated similar increased dyslipidaemia, hypertension, obesity and insulin resistance in both NHPT and PHPT populations (227). Other authors showed no impairment in insulin sensitivity and glucose tolerance in NHPT (228,229).

1.11 Management of normocalcemic hyperparathyroidism

The adequate management of NHPT is difficult to discern, as the long-term outcomes of therapeutic choices have not been sufficiently investigated. The Fourth International Workshop Guidelines for the Management of Asymptomatic Primary Hyperparathyroidism recommends clinical and biochemical follow-up of the disease (162). It is recommended to evaluate serum calcium, phosphate, alkaline phosphatase, 25(OH)D3, creatinine, blood urea nitrogen and PTH levels every year, and to perform a DXA scan every 1-2 years. The guidelines indicate that the surgical criteria for subjects with PHPT ought to be extended to those with the normocalcaemic form. Patients who develop kidney stones, nephrolithiasis, fracture, or bone loss would benefit from parathyroidectomy (162).

Medical Treatment. Few studies have evaluated the benefit of alendronate and cinacalcet in this condition and demonstrated the usefulness of these molecules in improving bone health and decreasing nephrolithiasis risk (230,231). The impact of medical therapies on cardiovascular and neurocognitive symptoms in NHPT are unknown.

Parathyroid Surgery. Follow-up of NHPT patients, after successful parathyroidectomy, showed improvements in BMD, nephrolithiasis, cardiovascular risk factors and quality of life (220,232,233). Despite these results, parathyroid surgery seems to be performed less frequently in NHPT compared to PHPT, due to the more complex morphology, poor sensitivity of preoperative imaging, and the lack of a sustained benefit than medical management.

CHAPTER 2: **RESEARCH PROJECT N°1**

CARDIOVASCULAR AUTONOMIC NEUROPATHY AS A NEW COMPLICATION OF POSTSURGICAL CHRONIC HYPOPARATHYROIDISM *

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2.1 Introduction

Conventional therapy for chronic hypoparathyroidism (hypoPT) includes activated vitamin D and/or calcium supplements, but this treatment does not fully replace the functions of PTH (80,234). Indeed, studies have shown that hypocalcemia and/or a lack of PTH may affect muscle function (235,236) and various aspects of QoL (80). These symptoms include physical (e.g., fatigue, muscle spasms, pain, and paresthesia), cognitive (e.g., "brain fog" and an inability to concentrate) and emotional complaints (e.g., depression and/or anxiety) (123). The nature of the impairment of QoL and its relationship to biochemical control or other aspects of the disease has not been well described. PTHrP and PTH/PTHrP receptors have been shown in the neurons, reactive astrocytes and endothelial cells of normal brain tissue (237), but their role is not fully understood.

There are few reports of peripheral neuropathy associated with hypoPT. The first case was reported in 1972 by Gay and Grimes in a 68-year-old man with hypoPT. Nerve conduction studies revealed a prolonged latency and decreased conduction velocity in the median and peroneal nerves, which improved after treatment with vitamin D and calcium (238). In 1984, Yang et al. reported a 4.2% prevalence of peripheral neuropathy in a cohort of 71 patients with hypoPT (239). In 2002, Goswami and colleagues (240) showed the occurrence of peripheral neuropathy in hypocalcemic states and its reversibility after the normalization of calcium level, proving the

importance of Ca2+ ion in the normal functioning of the peripheral axons. Patients with post-surgical hypoPT seem to show a two-fold increased risk of death after adjusting for age, sex, co-morbidities at the time of thyroidectomy and thyrotoxicosis as the indication for thyroidectomy. The cause of this increased mortality in hypoPT subjects is not clear but might be linked to lower PTH levels (241).

Cardiovascular autonomic neuropathy (CAN) is defined as an impairment of the cardiovascular autonomic control. Several studies in patients with diabetes have demonstrated that CAN is associated with increased fatigability and is a cause of increased mortality due to a high-risk of cardiac arrhythmias, silent myocardial ischemia and sudden death (242). Furthermore, the main clinical symptoms of CAN are usually represented by exercise intolerance, and resting tachycardia. The gold standard measures of cardiovascular autonomic function are the cardiovascular autonomic reflex tests (CARTs). These tests evaluate autonomic function through provocative physiological manoeuvres and by measuring the end-organ response; ie, heart rate and blood. Historically, they have been used to investigate different autonomic disorders (i.e. Parkinson's disease, Shy-Drager syndrome, Sjogren's syndrome, diabetic autonomic neuropathy, amyloid neuropathy) (243) now, they are largely used to evaluate CAN in diabetic population (244). In this setting, the tests have demonstrated to be sensitive, specific, reproducible, safe, and standardized (Supporting Table 2) (245,246).

No previous studies have evaluated CAN in hypoPT. Therefore, we aimed to investigate whether hypoPT is associated with CAN. In addition, we evaluated the relation between hypocalcemia, PTH levels and hyperphosphatemia with CAN.

2.2 Materials and methods

2.2.1 Study design and population

All subjects were enrolled from the outpatient clinic of the Endocrinology and Diabetes

unit of Campus Bio-Medico University from January to April 2018. We enrolled 51 consecutive subjects with postsurgical chronic hypoPT and 43 healthy subjects who underwent thyroidectomy (at least 6 months before enrolment) with adequate calcium, phosphate, magnesium and PTH levels (control group) without the use of any supplements. The indications for thyroidectomy included differentiated thyroid cancer (n=71; 75.5%), nontoxic goiter (n=19; 20.2%), and Graves' disease (n=4; 4.3%). The diagnosis of postsurgical chronic hypoPT was established at least 6 months postsurgery on the basis of clinical and biochemical features (low or inappropriately normal PTH levels with low calcium levels on at least two prior occasions separated by an interval of at least 30 days). All patients must have been on stable regimens of supplemental calcium carbonate and vitamin D for at least 3 months prior to enrolment. Subjects were excluded if they met any of the following criteria: (i) they suffered from diabetes mellitus, severe chronic liver or renal disease (glomerular filtration rate <30mL/min), Cushing's syndrome, sarcoidosis, rheumatic diseases (eg, systemic lupus erythematosus or rheumatoid arthritis), neurological disease, or had evidence of bone metastases; (ii) they took drugs that could interfere with calcium metabolism; (iii) they took antiarrhythmic drugs or other therapies that could interfere with cardiac rhythm; (iv) they took drug that could cause orthostatic hypotension such as diuretics, alpha blockers, or tricyclic antidepressants; (v) they took drugs that could interfere with the functioning of the nervous system; (vi) they took or had taken PTH(1–34) or PTH(1–84) in the past; (vii) they had serum magnesium levels below the lower limits or above the upper limits of normal on at least two prior occasions; or (viii) they have overt hyperthyroidism, indeed in subjects with overt hyperthyroidism it has been described an increase of sympathetic tone and a decrease of vagal regulation of heart (247).

Autonomic neuropathy evaluation. The following cardiovascular provocative tests have been performed to detect CAN: (i) heart rate (HR) response to deep breathing (expiratory-to-inspiratory ratio); (ii) HR response to lying-to standing test (30:15 ratio); (iii) HR response to the Valsalva manoeuvre (maximum HR during expiration/minimum HR after expiration ratio); and (iv) blood pressure response to

standing. Participants were considered to have CAN if they had one abnormal result in the HR tests (early CAN) or two or more abnormal results in the HR tests (definite CAN). The presence of orthostatic hypotension in addition to abnormal HR test results signifies advanced CAN. Therefore, the severity of CAN is also defined by the number of pathologic tests (244). The tests were performed using a Neuro Tester® (Meteda, San Benedetto del Tronto [AP], Italy). Age-related normal reference values are automatically provided by Neuro Tester according to Cardone (248). Orthostatic hypotension was defined by a reduction of systolic blood pressure of at least 20 mmHg or diastolic blood pressure of 10 mmHg within 3 min of standing (249). Smoking was not allowed in the 5 days before the neuropathy evaluation. The tests were performed in fasting status, and caffeine (coffee, tea, cola, energy drinks) and alcohol were forbidden on the day before the tests. During the procedure, the subjects were instructed to fully relax, stay awake, breathe regularly, and not to speak.

Assays. Intact parathyroid hormone (iPTH) was measured by an immunochemiluminometric assay using the automatic analyzer Modular E170 (Roche Diagnostics, Indianapolis, IN, USA). Normal serum iPTH levels ranged between 18 and 65 pg/mL. PTH serum concentrations between 10 and 18 pg/mL were arbitrarily defined as low PTH levels. PTH serum concentrations <10 pg/mL were arbitrarily defined as very low PTH levels. Serum calcium was measured by automated techniques, and normal levels ranged between 8.5mg/dL and 10.2mg/dL. Serum calcium was adjusted for albumin by the following formula: Alb-Ca=(0.8 [4.0 – patient's albumin]+serum calcium) (250). Serum phosphate (normal levels ranged between 2.4 and 4.4mg/dL), magnesium, and creatinine levels as well as thyroid function were also measured by automated techniques. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease–Epidemiology Collaboration (CKD-EPI) equation. Fasting blood samples were obtained in the morning, immediately before starting CAN evaluation (from 8:00 a.m. to 8:30 a.m.), and urinary calcium was measured by the colorimetric method.

Ethics. The study was conducted in compliance with the Declaration of Helsinki and

the International Conference on Harmonization Principles of Good Clinical Practice. The research protocol was approved by the Ethical Committee at the University Campus Bio-Medico of Rome and all participants gave informed consent allowing their anonymized information to be used for a data analysis.

2.2.2 Statistical analysis

The characteristics of the study were reported using descriptive statistics (mean and SD for continuous variables, proportions for categorical variables) in accordance with the diagnosis of hypoPT. We calculated the prevalence of CAN by hypoPT status and calcium concentrations using proportions, and the association between groups and CAN was evaluated using logistic regression models with nested controls. The association between hypoPT and CAN was evaluated using the odds ratio (OR) and 95% CI. The same analytical approach was used to calculate the association between hypocalcemia, PTH, and high phosphate concentration with CAN in the subgroup of patients with hypoPT. We used a multivariable logistic regression model to obtain the adjusted OR, taking into account the patients' age, sex, calcium excretion, eGFR, and levels of thyroid-stimulating hormone (TSH), PTH, phosphorus, 25-hydroxivitamin D, and duration of disease (all continuous variable except for the calcium that is categorical). The difference in the means of the calcium-phosphorus serum concentration product across CAN subgroups was evaluated using the Student's t test. We expected a prevalence of CAN (with at least one abnormal result on the HR test) of 12% in the control group (251) and 40% in the group with hypoPT. To declare the difference to be statistically significant with a type I error rate of 5% and a type II error rate of 20%, we would have needed to enrol 38 patients per group. All the analyses were performed using R version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/).

2.3 Results

Demographic and biochemical features. Demographic and biochemical data are

reported in Table 2. The study population consisted of a total 94 adults after total thyroidectomy (84 females and 10 males with a mean age of 50.7±6.4 years). All subjects were receiving levothyroxine therapy, and none of the patients had overt hyperthyroidism. We included 51 hypoPT patients (47 females and 4 males with a mean age of 52.6±13.6 years) and 43 healthy control subjects (37 females and 6 males with a mean age of 48.4±11.6 years). At the time of evaluation, all hypoPT patients were treated with calcitriol (0.65±0.39 mg/day) and calcium as carbonate (1468±807 mg). Compared with the control group, hypoPT patients had significantly lower Alb-Ca (8.5±0.6 versus 9.2±0.4 mg/dL, p<0.001), higher phosphate (4.5±0.9 versus 3.6±0.6 mg/dL, p<0.001), lower PTH (9.9±6.8 versus 46.7±18.3 pg/dL, p<0.001), and higher urinary calcium excretion (261.5±165.7 versus 127.1±83.7 mg/24 hours, p<0.001). No significant differences in the serum 25(OH)D3 levels, thyroid function, blood glucose, eGFR, blood pressure, use of antihypertensive, smoking habits, nephrolithiasis, or body mass index (BMI) were recorded between the two groups.

Table 2. General characteristics of the study population

	No HypoPT (n=43)	**HypoPT (n=51)**	**P-value**
Age (yrs), mean (SD)	48.4 (11.6)	52.6 (13.6)	0.107
Males (%)	14	8	0.534
Cause of Thyroidectomy (%)			
Goiter	16	23	0.352
Cancer	79	73	0.271
Graves' disease	5	4	0.842
Albumin-corrected serum calcium (mg/dl), mean (SD)	9.2 (0.4)	8.5 (0.6)	< 0.001
Serum PTH (pg/dl), mean (SD)	46.7 (18.3)	9.9 (6.8)	< 0.001
Serum phosphate (mg/dl), mean (SD)	3.6 (0.6)	4.5 (0.9)	< 0.001
Serum 25(OH)D3 (ng/ml), mean (SD)	26.6 (9.8)	26.3 (10.6)	0.897

eGFR (ml/min/1.73mq), mean (SD)	97.1 (33.8)	100 (25.6)	0.637
TSH (ng/dl), mean (SD)	0.6 (1.2)	1 (1.1)	0.072
Urinary calcium (mg/24h), mean (SD)	127.1 (83.7)	261.5 (165.7)	< 0.001
Mg (mg/dl) mean (SD)	1.9 (0.2)	2.0 (0.2)	0.913
Blood glucose (mg/dl), mean (SD)	94.4 (9.6)	91.8 (7.8)	0.232
Smokers (%)	28	17.6	0.235
Nephrolithiasis (%)	2.3	7.8	0.231
Systolic blood pressure (mmHg) mean (SD)	120(8.8)	120 (13)	0,764
Diastolic blood pressure (mmHg) mean (SD)	76 (7.3)	73 (7.3)	0,102
BMI (kg/m2) mean (SD)	24.9(3.1)	24.5 (4.2)	0.609
Use of antihypertensive (%) **ACE-inhibitors** **Angiotensin-receptor blockers**	14 7 7	14 10 4	0.972
Duration of hypoparathyroidism (years) mean (SD)		9.1 (8.4)	
CAN (%)	23	78	< 0.001
Definite CAN (%)	2	43	<0.001

CAN evaluation. The prevalence of CAN was 23% among the patients without hypoPT and 78% among the patients with hypoPT (OR 11.48; 95% CI, 4.48 to 32.17). The rate of abnormal tests in the study population has been reported in Table 3. The average calcium concentration in the group with hypoPT was 8.5 mg/dL (SD 0.6), and the proportion of people with calcium <8.5 mg/dL was 43.1%. Compared to the participants without hypoPT, those with hypoPT but with normal serum calcium concentrations (≥8.5 mg/dL) had a prevalence of early CAN of 72.4%, and those patients with serum calcium concentrations <8.5 mg/dL had a prevalence of early CAN of 86.4%. The corresponding figures for definite CAN were 2.3% in the group without hypoPT, 24.1% in the group with hypoPT and without hypocalcemia, and 59.1% in the

group with hypoPT and hypocalcemia (Figure 5). No advanced CAN has been identified in the study population. In the group with hypoPT, OR of having early CAN among patients with hypocalcemia is not significantly increased compared to subjects without hypocalcemia (OR 2.41; 95% CI, 0.56 to 10.44). The OR for definite CAN in patients with hypocalcemia compared with normocalcemic patients with hypoPT, was 4.54 (95% CI, 1.36 to 15.11). In the hypoPT group, the prevalence of subjects with PTH <10 pg/dL (very low PTH) was 49%. The prevalence of early CAN in participants with very low PTH was 88% and the prevalence of early CAN in participants with low PTH concentration (PTH between 10 and 18 pg/dL) was 69%. No association was found between early CAN and very low PTH concentration (88% versus 69%; OR 3.26; 95% CI, 0.75 to 14.12). No association was found between definite CAN and very low PTH concentration (48% versus 31%; OR 2.08; 95% CI, 0.66 to 6.52). Similar results were found with respect to phosphate serum concentration, as the prevalence of CAN in participants with and without high phosphate concentration was 76% and 80%, respectively (OR 0.8; 95% CI, 0.21 to 3.07), and the corresponding prevalence of definite CAN was 43% and 37%, respectively, with OR 1.3 (95% CI, 0.41 to 4.05). The calcium*phosphorus average product in hypoPT participants was 37.5mg2/dL2, and it was not different among people with early CAN compared with those without early CAN (36.9 versus 39.7mg2/dL2; p¼0.35). There was no difference comparing participants with and without definite CAN (38.1 versus 37.1mg^2/dL^2; p=0.59). The association between low calcium concentration and definite CAN was confirmed after adjustment for potential confounders (see Table 4) with OR 13.62 (95% CI. 2.12 to 149.84). Furthermore, after adjustment for potential confounders, there is also a positive association between disease duration and definite CAN with OR 1.1 (95% CI, 1.00 to 1.24).

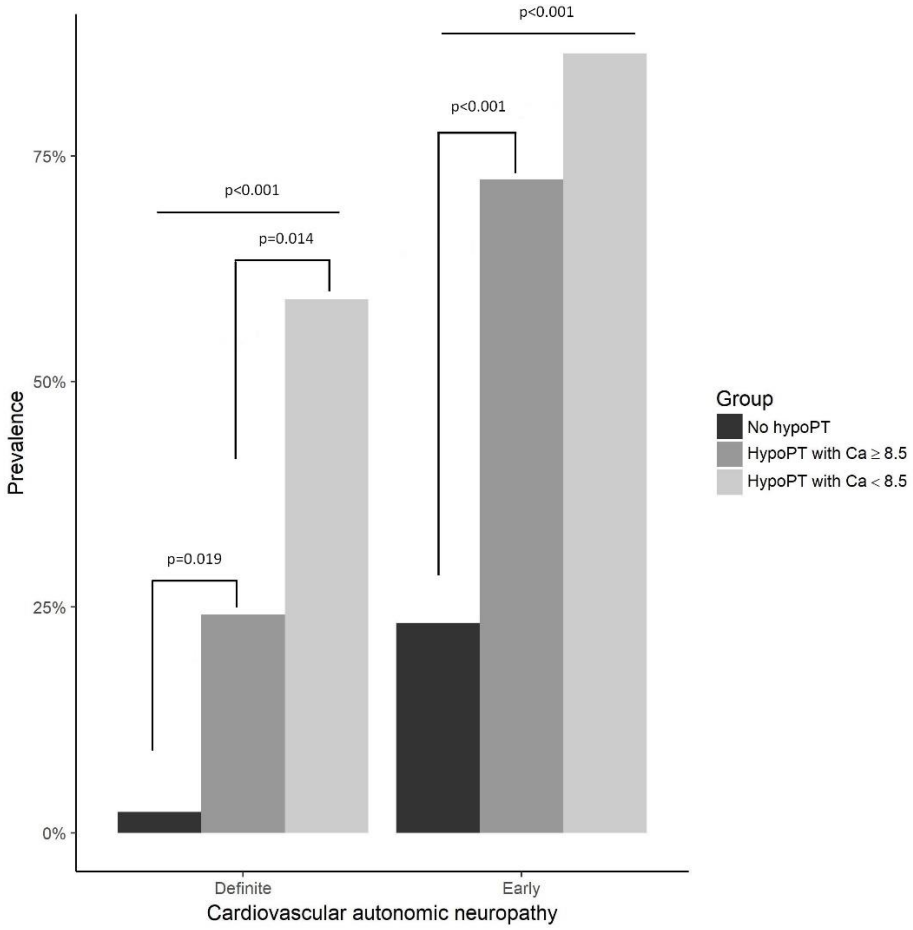
Table 3. Prevalence of Abnormal Tests in the Study Population

	% Deep breathing (n.)	% Valsalva maneuver (n.)	% Lying to standing (n.)	% Orthostatic hypotension (n.)
Hypoparathyroidism *overall* (n=51)	22 (11)	41 (21)	61 (31)	0
Hypoparathyroidism and *calcium<8.5 mg/dl* (n=22)	27 (6)	64 (14)	68 (15)	0
Hypoparathyroidism and *calcium ≥ 8.5 mg/dl* (n=29)	17 (5)	24 (7)	55(16)	0
Controls (n=43)	0	12 (5)	14 (6)	0

Table 4. Definite CAN: Multivariable Model Adjusted for the Reported Variables

	Odds Ratio	**Lower 95% CI**	**Upper 95% CI**
Albumin-adjusted calcium < 8.5 mg/dl	13.62	2.12	149.84
PTH serum concentration	0.99	0.85	1.15
Phosphate serum concentration	2.08	0.78	6.43
Urinary calcium	1.00	0.99	1.00
TSH	0.69	0.29	1.49
25(OH)D3	1.08	0.99	1.19
Age	1.02	0.95	1.10
Sex	14.31	0.36	744.01
Duration of disease	1.10	1.00	1.24

Figure 5. Prevalence of CAN by hypoparathyroidism and serum calcium concentration.



2.4 Discussion

For the first time, we showed that chronic postsurgical hypoPT is associated with CAN and that hypocalcemia seems to affect its severity. CAN is defined as the impairment of the autonomic control of the cardiovascular system and different approaches have been used to diagnose this condition in practice or research. We performed the cardiovascular autonomic reflex tests to investigate the integrity of the autonomic nervous system because they are sensitive, specific, reproducible, safe, standardized (245,246) and eminent guidelines recommended their use as the gold standard for clinical autonomic testing (244).

Parasympathetic function seems to be primarily evaluated by cardiovascular autonomic

reflex tests that evaluate HR variability, whereas sympathetic function seems to be better studied by blood pressure variability (243). In particular, the deep breathing test evaluates the physiological sinus arrhythmia during quiet respiration that is under control of parasympathetic function (252); the lying to standing test evaluates tachycardia followed by bradycardia that occurs after standing (vagal and baroreflex response); the Valsalva manoeuvre assesses the cardiovagal function stimulates by the increase in intrathoracic pressure; the orthostatic hypotension investigates the sympathetic function (242).

In our population, 78% of hypoPT subjects had at least one positive HR variability test, although none of the subjects had a pathologic result to the orthostatic hypotension test, suggesting that there might be significant impairment of parasympathetic activity.

Lack of PTH and/or hypocalcemia might be causative factor(s) for the increased rate of CAN in patients with hypoPT. Indeed, some studies have found a relationship between excess PTH and peripheral nerve conduction or autonomic system impairment. PTH plays a major role in the genesis of peripheral nerve dysfunction in chronic renal failure, probably mediated by the PTH-induced accumulation of calcium in the peripheral nerves (253). Furthermore, Barletta and colleagues (254) showed that chronic PTH excess may determine a modulation of the adrenergic control of circulation in normotensive patients with asymptomatic hyperparathyroidism. The authors found an increase of sympathetic tone in half of the hyperparathyroidism patients and, interestingly, parathyroidectomy restored the physiological rhythm of sympathovagal balance.

PTH and PTHrP could also exert positive inotropic effects on the heart and positive chronotropic and vasodilator effects due to their influence on coronary flow and HR (255).

However, in our study population, we did not find a correlation between the PTH levels and the risk of CAN. It is likely that the disease (chronic hypoparathyroidism) more than the lack of PTH can play a role in determining autonomic dysfunction.

On the other hand, the worsening of CAN in our population with lower calcium levels may highlight the importance of calcium in the development of autonomic neuropathy. It has been demonstrated that low calcium level determines an increase of neuromuscular irritability and hypocalcemia increases the probability of triggering action potentials in the membrane of a neuron (80,256).

In subjects with chronic hypoPT, European Society of Endocrinology and Endocrine Society guidelines (234) suggest maintaining serum calcium concentrations slightly below normal (ie, no more than 0.5 mg/dL below normal) or in the low normal range to reduce the long term incidence of chronic kidney complications, namely, nephrocalcinosis and renal function impairment. However, no strong evidence supports this therapeutic approach, and whereas it could reduce the risk of hypercalciuria, it may increase the risk of CAN. Indeed, although 35 of 51 (69%) hypoPT subjects met the recommended Alb-Ca concentrations as suggested by the guidelines (Alb-Ca target: 8 to 9 mg/dL), 14 of 35 (40%) subjects presented with early CAN, and 12 of 35 (34%) subjects presented with definite CAN.

Moreover, CAN is a significant and independent predictor of morbidity and mortality in diabetes (242). Although it is likely that pathogenesis of CAN is markedly different between diabetes and hypoPT, the presence of CAN might partly explain the increase of mortality in hypoPT, that has not been elucidated yet (241). Therefore, maintaining a serum calcium level below the normal range might not be the appropriate goal for all patients with chronic hypoPT. However, we cannot exclude that the presence of CAN in hypoPT might be related to calcium fluctuations, especially because ionized calcium levels may be affected by the deep breathing and the Valsalva maneuver. Because free ionized calcium and pH-values were not measured in our study, this possibility cannot be directly evaluated. Nevertheless, the high rate of abnormal lying to stand test in the subjects with hypoPT (61% versus 14% in the control group, $p<0.001$) would suggest additional mechanisms, because this provocative test is not known to change pH and free ionized calcium level.

Interestingly, CAN might also explain the postoperative fatigue associated with hypoparathyroidism. Indeed, subjects with CAN often complain symptoms such as exercise intolerance and weakness (257), as well as patients with hypoPT do (80). Our hypothesis seems to be supported by previous studies that have demonstrated a relationship between fatigue and autonomic dysfunction during different chronic diseases, namely Parkinson's disease (258) multiple sclerosis (259) and chronic fatigue syndrome (260). Evaluations of fatigue have not yet been performed, and therefore, we are conducting further analyses to investigate the relationship between fatigue and CAN.

Finally, we found that the duration of disease is associated with the risk of developing CAN. This finding reflects what showed in other chronic disease, as diabetes, where the duration of disease is one of the main predictors for CAN (257).

Our study has some limitations. We did not perform cardiac scintigraphy, echocardiograms (useful in identifying diastolic dysfunction), or cardiac magnetic resonance (able to detect early stages of CAN via myocardial torsion). Moreover, we did not evaluate the catecholamine levels in the blood or 24-hour urine collection and we did not measure the free ionized calcium.

In conclusion, hypoPT is associated with CAN, and hypocalcemia seems to affect its severity. The presence of CAN could explain fatigue and the increase of mortality during hypoPT. Larger and prospective studies are needed to confirm our findings.

CHAPTER 3: **RESEARCH PROJECT N°2**

CARDIOVASCULAR AUTONOMIC NEUROPATHY AS A CAUSE OF FATIGUE IN CHRONIC HYPOPARATHYROIDISM *

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3.1 Introduction

Chronic hypoPT is a pathological condition that is characterized by low serum calcium levels with low or inappropriately normal serum PTH levels. Conventional therapy consists of activated vitamin D and/or calcium supplements. However, hypoPT patients often complain of symptoms suggestive of a reduced QoL (121), namely fatigue, muscle spasms, pain, "brain fog", an inability to concentrate, depression and anxiety (123,261), despite adequate supplementation with calcium/vitamin D and the serum calcium concentration in the therapeutic range. Furthermore, postsurgical chronic hypoPT patients seem to have a twofold increased risk of death after adjusting for age, sex and co-morbidities (241), although the causes of this increased mortality are unknown. Cardiovascular autonomic neuropathy (CAN) is an impairment of cardiovascular autonomic control and is associated with increased fatigue and mortality in subjects with diabetes (242). Recently, we demonstrated that postsurgical hypoPT was strongly associated with CAN (262), and we hypothesized that CAN might be one cause of the fatigue and impaired QoL described by these patients.

The aim of our investigation is to test whether CAN is associated with fatigue and impaired QOL measures in patients with postsurgical chronic hypoPT.

3.2 Materials and methods

3.2.1 Study design and population

Study population. The study includes 48 consecutive subjects with postsurgical chronic hypoPT. As a control group, we enrolled 38 subjects without any calcium metabolism disorders who underwent thyroidectomy (at least 6 months prior to enrolment). The subjects were recruited from the endocrinology outpatient clinic of Campus Bio-Medico University, Rome, Italy from January to April 2018. The indications for thyroidectomy were: differentiated thyroid cancer (n = 63, 73.2%), nontoxic goiter (n = 20, 23.3%), and Graves' disease (n = 3, 3.4%). The diagnosis of postsurgical chronic hypoPT was established at least 6 months post surgery based on the presence on two different occasions of low calcium levels with low or inappropriately normal PTH levels. All patients were on stable regimens of supplemental calcium carbonate and vitamin D for at least 3 months prior to enrolment.

The exclusion criteria were as follows: (1) the presence of diabetes mellitus, severe chronic liver, or renal disease (glomerular filtration rate <30 mL/min), Cushing's syndrome, sarcoidosis, rheumatic diseases (e.g., systemic lupus erythematosus or rheumatoid arthritis), neurological disease, or evidence of bone metastases; (2) use of drugs that could interfere with calcium metabolism; (3) history of any cardiovascular diseases (4) use of antiarrhythmic drugs or other therapies that could interfere with cardiac rhythm; (5) use of drugs that could cause orthostatic hypotension, such as diuretics, alpha-blockers, or tricyclic antidepressants; (6) use of drugs that could interfere with the functioning of the nervous system; (7) current or previous use of PTH (1–34) or PTH (1–84); (8) serum magnesium levels below the lower limits or above the upper limits of normal on at least two prior occasions; or (9) overt hyperthyroidism.

Autonomic neuropathy evaluation. CAN was detected using a Neuro Tester® [Meteda, San Benedetto del Tronto (AP), Italy]. Four different tests were performed as follows: (1) heart rate (HR) response to deep breathing (expiratory-to-inspiratory ratio), (2) HR response to the lying-to-standing test (30:15 ratio), (3) HR response to

the Valsalva manoeuvre (ratio of maximum HR during expiration/minimum HR after expiration), and (4) blood pressure response to standing. Each test has been repeated three time and Neuro Tester automatically provides the mean score value. Age-related normal reference values are automatically provided by Neuro Tester, according to Cardone C (248). Participants were considered to have early CAN if they had one abnormal result in the HR tests, definite CAN if they had two or more abnormal results in the HR tests, and advanced CAN if they had orthostatic hypotension in addition to abnormal HR test results (244). Smoking was forbidden in the 5 days prior to the neuropathy evaluation. The tests were performed in a fasting status, and caffeine (coffee, tea, cola, or energy drinks) and alcohol were not allowed on the day before the tests.

Quality of life evaluation. QoL was evaluated using the SF-36 (version 1) and Functional Assessment of Chronic Illness Therapy (FACIT)- Fatigue score (version 4). The SF-36 is a validated instrument for the evaluation of QoL. The survey assesses patient health across 36 items that are grouped into eight domains: bodily pain, general health perceptions, mental health, physical functioning, role limitations due to emotional functioning (RE), role limitations due to physical functioning (RP), social functioning, and vitality. From the eight individual subscales, two component summary scores are generated for the physical component summary (PCS) and mental component summary (MCS) scores (263). Several validated tools are available for the measurement of fatigue, but no gold standard has been defined. We used the FACIT Measurement System, which is a collection of health-related QoL questionnaires that are designed for the management of chronic diseases, and the subscale of the FACIT-fatigue. The FACIT-fatigue is a short, 13-item fatigue subscale that measures an individual's level of fatigue during usual daily activities over the past week. Each question is scored on a four-point Likert scale (4 = not at all fatigued to 0 = very much fatigued), with a higher total score indicating a better QoL (264). The questionnaires were administered in a blinded manner by a trained student (AMDT) before performing the CAN evaluation.

Assays. iPTH was measured with an immunochemiluminometric assay using the Modular E170 automatic analyzer (Roche Diagnostics, Indianapolis, IN, USA), with normal levels ranging between 18 and 65 pg/mL. Serum calcium was measured by automated techniques (normal levels ranged between 8.5 and 10.2 mg/dL) and was adjusted for albumin using the following formula: Alb-Ca = (0.8 [4.0 − patient's albumin] + serum calcium) (250). The serum phosphate (normal levels ranged between 2.4 and 4.4 mg/dL), magnesium, and creatinine levels and thyroid function were also measured using automated techniques. The estimated glomerular filtration rate was calculated using the CKD-EPI equation. Fasting blood samples were obtained in the morning immediately before starting the CAN evaluation (from 8:00 to 8:30 A.M.).

3.2.2 Statistical analysis

We compared the characteristics of the participants with and without hypoPT using descriptive statistics (means and standard deviations for continuous normally distributed and median and interquartile range for continuous non-normally distributed variables, and proportions for categorical variables). Differences were tested using the T-test for continuous normally distributed variables, Wilcoxon–Mann–Whitney test for non-normally distributed, and the $\chi 2$ test for proportions. Then, we created three groups according to the presence of CAN (no CAN, early CAN, and definite CAN). Differences among these groups were evaluated using analysis of variance or Kruskal–Wallis and Dunn's test as appropriate for continuous variables and the $\chi 2$ test for proportions. For the patients with hypoPT, the association between CAN and QoL (fatigue score, PCS, MCS, and total SF-36 score) was evaluated using linear regression models with "no CAN" as the reference group. The models were adjusted for potential confounders selected and the results of the univariate analyses (age, calcium concentration, calcium supplementation, calcitriol supplementation, duration of disease and TSH). All analyses were performed using R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

3.3 Results

General characteristics of the whole study population. We enrolled 86 participants, including 48 with a diagnosis of hypoPT and 38 control subjects. The mean age of the overall population was 51.2 years (SD: 12.9), and 9% of the subjects were male. No differences were found between the controls and the hypoPT subjects in terms of age, sex, TSH and 25 (OH) vitamin D levels (Table 5).

Table 5. General characteristics of the overall population

	No HypoPT N: 38	**HypoPT N: 48**	**P-value**
Age, (yrs, mean (SD)	49.2 (11.3)	52.9 (13.9)	0.178
Male sex, (%)	11	8	1
Serum calcium (mg/mL), mean (SD)	9.1 (0.3)	8.4 (0.6)	<0.001
Serum PTH (pg/dL), median (IQR)	42.8 (22.8)	8.45 (10.4)	<0.001
Serum phosphate (mg/dL), mean (SD)	3.6 (0.6)	4.4 (0.9)	<0.001
25 OH Vitamin D (ng/mL)	27.2 (10.1)	26.1 (10.9)	0.618
TSH (ng/dL), mean (SD)	0.63 (1.3)	1.02 (1.08)	0.142
Calcium supplementation (mg/day)	0 (0)	1464.6 (813.3)	
Calcitriol supplementation (mcg/day)	0 (0)	0.64 (0.4)	

PCS, median (IQR)	350 (138.8)	297.5 (145.6)	0.054
MCS, median (IQR)	268.3 (150.5)	286.7 (162)	0.952
SF-36 total, median (IQR)	604.3 (252.5)	589.2 (302.9)	0.444
Fatigue score, median (IQR)	44.5 (9)	38.5 (12.3)	0.031

General characteristics of the hypoPT population. Of the 48 hypoPT participants, 10 (21%) had no CAN, 19 (39%) had early CAN, 19 (39%) had definite CAN and no subject experienced advanced CAN. No differences were noted among these groups in age, sex, and the serum calcium, PTH, phosphate, vitamin D, and TSH concentrations. In addition, no differences were noted in calcium supplementation, although the dosage of calcitriol supplementation slightly differed across the groups (no CAN 0.25 IQR: 0.25 mcg/day; early CAN 0.5 IQR: 0.12 mcg/day; and definite CAN 0.75 IQR: 0.63 mcg/day, P = 0.016) (Table 6).

Table 6. General characteristics of the hypoPT population

	No CAN **N: 10**	**Early CAN** **N: 19**	**Definite CAN** **N: 19**	**P-value**
Age, (yrs, mean SD)	51.7 (16.5)	53.3 (13.6)	53.1 (13.7)	0.958
Male sex (%)	20	0	11	0.163
Serum calcium (mg/mL), mean (SD)	8.6 (0.5)	8.5 (0.6)	8.2 (0.6)	0.181
Serum PTH (pg/dL), median (IQR)	11.5 (4.3)	7.3 (11.3)	5.5 (11)	0.265

Serum phosphate (mg/dL), median (IQR)	4.5 (1.1)	4.1 (0.8)	4.7 (0.8)	0.159
25 OH Vitamin D (ng/mL)	22.3 (9.2)	27.2 (10.9)	27 (11.7)	0.469
TSH (ng/dL), mean (SD)	0.9 (0.8)	1 (1.3)	1.1 (1)	0.932
Calcium supplementation (mg/day)	1010 (699.9)	1515.8 (803.6)	1652.6 (825.4)	0.121
Calcitriol supplementation (mcg/day), median (IQR)	0.25 (0.25)	0.5 (0.12)	0.75 (0.63)	0.016
PCS, median (IQR)	355 (42.4)	290 (158)	270 (114)	0.054
MCS, median (IQR)	345 (126)	293 (177)	241 (137)	0.257
SF-36 total, median (IQR)	687 (197)	588 (362)	500 (256)	0.093
Fatigue score, median (IQR)	46 (2.5)	42 (12)	45 (7.5)	0.005

Fatigue and quality of life evaluation. Compared with controls, the participants with hypoPT had lower PCS (350 IQR: 138.8 vs 297.5 IQR:145.6, P =0.054) and fatigue scores (44.5 IQR: 9 vs 38.5 IQR: 12.3, P = 0.031), whereas no differences were found in the MCS and total SF-36 scores (Table 5). In the hypoPT subjects, we did not record any differences in the MCS, PCS, and total SF-36 scores between the subjects with no

CAN, early CAN and definite CAN (Table 6). These findings were also confirmed by linear regression models using the absence of CAN as the reference group after adjustment for different confounding factors (Table 7). With respect to the eight SF-36 domains, no differences were found among the groups after adjustment for different confounding factors (data not shown). We recorded a significant difference in the fatigue score among the no CAN, early CAN, and definite CAN groups (no CAN: 46 IQR: 2.5; early CAN 42 IQR: 12; and definite CAN 45 SD: 7.5; P = 0.005) (Table 2). Dunn's post hoc analysis documented that with respect to the participants without CAN, only those with definite CAN had a statistically significant lower fatigue score (P = 0.001, Figure 6). These results were confirmed by linear regression models adjusted for potential confounders (early CAN: β −5.67, P = 0.063; definite CAN: β −9.55, P = 0.005) (Table 7).

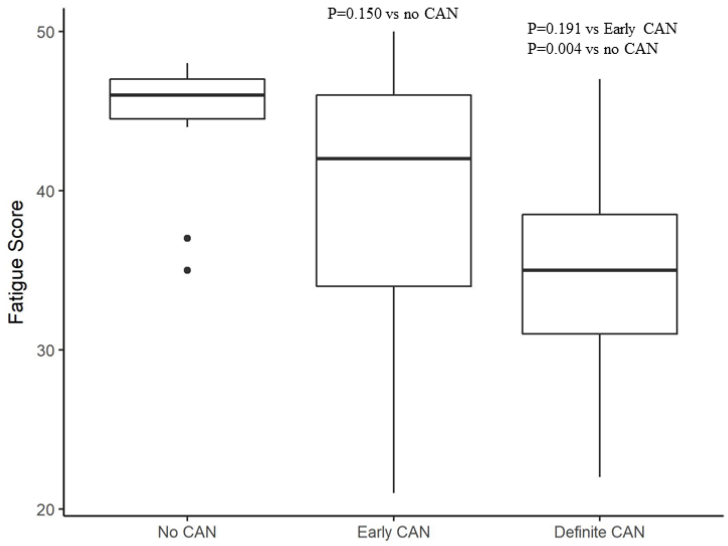
Table 7. Linear regression models of the association between CAN with fatigue and quality of life using the absence of CAN as the reference group

	β crude (P)	β adjusted[a] (P)
Fatigue score		
Early CAN	-5.24 (0.06)	-5.67 (0.063)
Definite CAN	-9.30 (0.002)	-9.55 (0.005)
PCS		
Early CAN	-53.89 (0.118)	-51.30 (0.150)
Definite CAN	-81.26 (0.020)	- 50.23 (0.195)
MCS		

Early CAN	-22.73 (0.548)	-27.92 (0.469)
Definite CAN	-46.53 (0.222)	-45.28 (0.284)
Total SF-36		
Early CAN	-76.62 (0.253)	-79.22 (0.254)
Definite CAN	-127.79 (0.060)	-95.51 (0.208)

[a]Models adjusted for age, calcium concentration, TSH, calcium supplementation, calcitriol supplementation and duration of disease

Figure 6. Changes in the fatigue score according to the CAN classification



3.4 Discussion

We showed that CAN might be associated with fatigue in subjects with chronic postsurgical hypoPT. Patients with hypoPT, and particularly patients with postsurgical hypoPT, often experience symptoms suggestive of a poor QoL. The most common

symptoms are fatigue, muscle cramps, paresthesia, pain, poor memory, a lack of concentration, depression, and anxiety (261). However, whether the symptoms are related to the absolute serum calcium level, the stability and variability of serum calcium, or PTH deficiency is unknown (261,265). Studies focused on QoL in patients with chronic hypoPT have not shed light on a biological mechanism that can explain the QOL impairments (121,127,265–268).

In our evaluation, hypoPT subjects experienced higher fatigue levels and showed a slight reduction in PCS than controls that did not reach statistical significance. A previous study have showed a lower PCS score in postsurgical hypoPT patients than in subjects who underwent thyroidectomy, whereas no differences were found in the MCS score (121). Therefore, Sikjaer et al. (121) supported the hypothesis that the QoL impairment in hypoPT subjects was not related to the chronicity of the pathological status and that its pathogenesis needed to be fully elucidated.

Recently, we demonstrated that CAN was associated with hypoPT (262). CAN is an impairment of cardiovascular autonomic control, resulting in a reduction in HR variability. The symptoms of CAN are resting tachycardia, exercise intolerance, orthostatic hypotension, dizziness, and sudden death (257). Because fatigue is one of the most common symptoms of hypoPT and may be related to the heart dysfunction, we hypothesized that fatigue could be the result of CAN. Indeed, previous studies have demonstrated a relationship between fatigue and autonomic dysfunction in different chronic diseases. In particular, Nakamuta et al. demonstrated that autonomic dysfunction, including cardiac sympathetic denervation, was associated with fatigue in patients with Parkinson's disease (258). Merkelbach et al. evaluated cardiovascular autonomic dysregulation and fatigue in patients with multiple sclerosis and showed that autonomic disturbances might contribute to fatigue symptoms in a multiple sclerosis subgroup (259). Furthermore, autonomic dysfunction has been strongly associated with fatigue in subjects with chronic fatigue syndrome (260), and the physical activity index is a significant predictor of autonomic neuropathy in this pathological condition (269).

In agreement with these findings, we found a significant association between definite CAN and fatigue. We can speculate that fatigue in hypoPT patients is a result of the inability of the heart to adjust its function to the physical activity level of the subject.

The pathogenesis of CAN in hypoPT needs to be elucidated. In our population, no differences were found in the serum calcium, PTH, phosphate, vitamin D, and TSH concentrations among the subjects with no CAN, early CAN, and definite CAN. The use of calcium supplementation was the same among the groups. However, the calcitriol dosage showed a slight increase among the groups, although this difference was not clinically significant. Previous studies on QoL and hypoPT seem to show an increase in well-being after starting PTH replacement therapy (266–268,270), but whether the use of PTH analogs can improve CAN and fatigue needs to be evaluated.

This study has some limitations. Both SF-36 and FACIT are validated but are not disease-specific questionnaires (to date, no disease-specific questionnaires are available). We did not perform physical tests to evaluate fatigue.

In conclusion, CAN may explain fatigue in subjects with chronic postsurgical hypoPT, which is a common complaint of this pathological condition. Further and larger studies are needed to confirm our findings.

CHAPTER 4: **RESEARCH PROJECT N°3**

DXA-BASED BONE STRAIN INDEX: A NEW TOOL TO EVALUATE BONE QUALITY IN PRIMARY HYPERPARATHYROIDISM*

**This manuscript is actually under revision.*

4.1 Introduction

Primary hyperparathyroidism (PHPT) is a common endocrine disorder. It is characterised classically by hypercalcemia and high or inappropriately normal (unsuppressed) PTH levels. Major complications of PHPT include nephrolithiasis, osteoporosis, and fragility fracture (271). Although increased fractures risk at both vertebral and non-vertebral sites are well established clinically, BMD is typically reduced at the distal 1/3 forearm, a primarily cortical bone site, with relative preservation of the lumbar spine, a predominantly trabecular site (194,272). HRpQCT has resolved these discrepant findings by demonstrating at the microarchitectural level that trabecular bone is also affected adversely in PHPT (158). However, as primarily a research tool, HRpQCT is not widely available. Other more readily available indices are needed to evaluate bone quality in the clinical setting.

Until recently, TBS has been the main DXA-based measure of bone quality in PHPT. Studies on postmenopausal women with PHPT showed a reduction in TBS values (157,273), at a time when the lumbar spine DXA value favored are much more positive assessment. Questions remain, however, as to whether vertebral fracture can be readily detected in this clinical setting (274).

Bone Strain Index (BSI) is a new DXA-derived skeletal parameter of deformation that is based on Finite Element Method (FEM). It can be applied both to lumbar and femoral DXA scans (275). Recent clinical studies showed the usefulness of BSI as a complementary tool, together with other clinical risk factors, to identify patients at risk of fracture (276,277) and to better characterize young patients affected by secondary

osteoporosis (278,279). Patients treated with teriparatide experienced an improvement of BSI over the time (280).

The aim of this study was to evaluate the lumbar spine and femoral BSI in subjects with PHPT compared to controls.

4.2 Material and methods

4.2.1 Study design and population

Study design and population. This study consisted of 44 subjects with primary hyperparathyroidism and 39 controls. Subjects with PHPT were consecutively enrolled from September 2017 to February 2019, at Campus Bio-Medico University of Rome. PHPT was defined as elevated or unsuppressed PTH concentrations and persistently elevated total, albumin-corrected, or ionized serum calcium levels (at least 2 different determinations, at least 3 months apart). Exclusion criteria were the following: any other condition that can affect bone and calcium metabolism; use of drugs affecting bone and calcium metabolism; history of possible high-energy VFx; metabolic bone diseases such as Paget disease and osteogenesis imperfecta. Control subjects were recruited from a historical cohort (216) who did not have any known impairment of mineral homeostasis as defined by normal calcium, phosphate, and PTH concentrations. They were consecutively recruited using the aforementioned criteria from the outpatient clinic of endocrinology at Campus Bio Medico University of Rome, where they were referred for unrelated diseases (eg, thyroid nodules with euthyroidism). Before enrolment, no control subject had ever had a BMD test. In all control subjects with osteoporosis and/or fragility fractures, secondary causes of osteoporosis were ruled out.

Biochemical analysis. Fasting blood samples were collected for the following: serum total calcium (normal, 8.4-10.2 mg/ dL) albumin (normal 3.2-4.6 g/dl), serum phosphate (normal, 2.3-4.7 mg/ dL) creatinine (normal 0.55-1.2 mg/dl) and 25 OH

vitamin D (normal, 30-100 ng/mL), were measured by automated methods. Calcium values were corrected for albumin concentration. Ionized serum calcium (normal, 1.13-1.32 mmol/L) was measured by a potentiometric method on GEM PREMIER 4000 analysers (Werfen, Le Pré-Saint-Gervais, Franceand. Intact PTH (normal, 14-72pg/mL) was measured by an immunochemiluminometric assay using a Modular E170 automatic analyser (Roche Diagnostics, Indianapolis, IN, USA). Serum levels of CTX were assayed by the β-CrossLaps (ECLIA; β-CrossLaps/Serum, Roche Diagnostics, Basel, Switzerland), which uses 2 monoclonal antibodies against β-crosslinked CTX according to the manufacturer's protocol. Serum levels of P1NP were analysed by sensitive electrochemiluminescent detection technology and was formatted for the Cobas Total P1NP (Roche Diagnostics) automated analyser.

Dual-energy X-ray absorptiometry. We measured BMD by DXA at the lumbar spine (L1-L4), total hip, femoral neck, and nondominant forearm (one-third distal radius) (Hologic Discovery QDR Instrument, MA, USA, version 13.3:3). Data were reported for absolute BMD and T-scores values (SD difference from mean values of sex matched young healthy individuals). All scans were performed according to the International Society for Clinical Densitometry (ISCD) guidelines (281). Fractured vertebrae and vertebrae with structural changes were excluded from the analysis (T-score difference with the adjacent vertebra > 1.0). TBS was automatically derived from the same region of lumbar spine DXA by dedicated software (Insight TBS, Medimaps Groupe, Geneve, Switzerland).

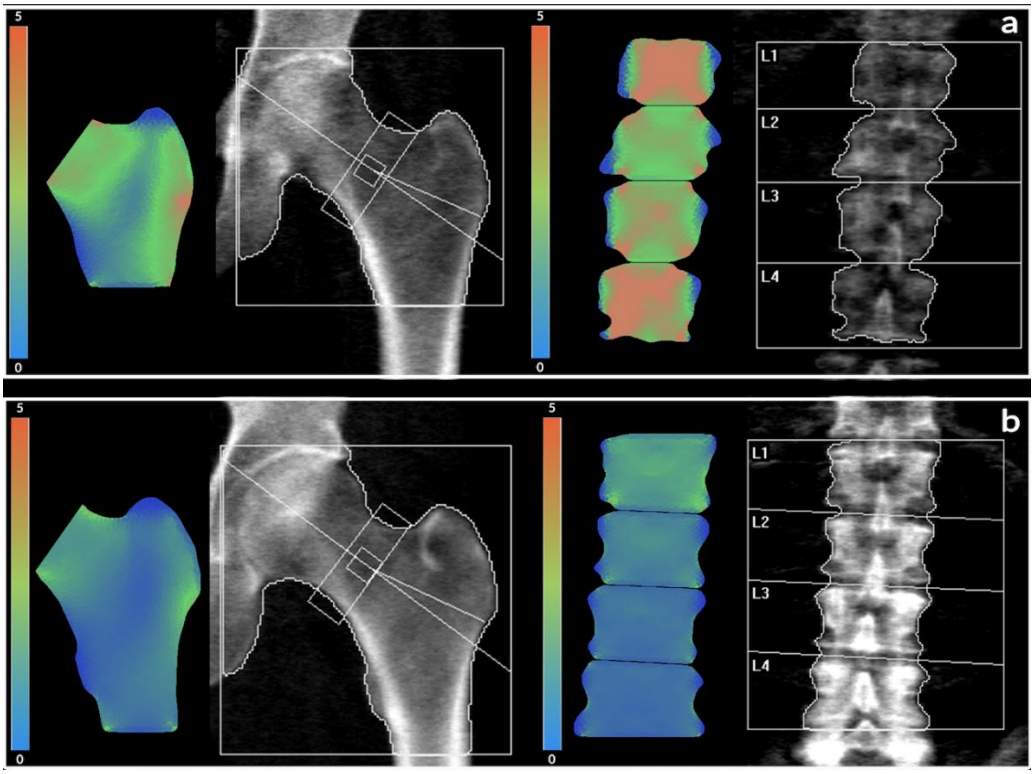
Bone Strain Index. For BSI analysis, raw data from the DXA image were sent to a separate workstation in which the BSI software (Tecnologie Avanzate s.r.l., Torino, Italy) is installed. For the lumbar spine, BSI computation was determined by dividing each vertebra of the lumbar scan into several triangles following the contour provided by DXA software mapping (282). The DXA lumbar image was analyzed by a pattern drawn with the load applied to the upper plate and the constraints to the lower plate of each vertebra (283). At the femoral site, BSI was calculated on the premise of a lateral fall, with force applied to the greater trochanter and the constraints applied to the

femoral head and shaft (284). In both spine and femur, stiffness of the elements was defined by the empirical relations described by Morgan et al.(285), at each anatomic site. The resulting amount of strain, presented graphically in different colors, led to identification of areas with the higher strain concentration. The BSI value represents the average equivalent strain in the regions defined by DXA analysis, with the assumption that a higher strain level (high BSI) indicates higher fracture risk. (Figure 7) FEA computation was automatically performed on the computer by dedicated software both for lumbar spine and hip, using the same region of interest that is utilized for BMD calculations (275,286). Each BSI examination takes about 5 seconds.

Figure 7. Femoral and Lumbar Spine BSI images in a subject with hyperparathyroidism (a) and in a control subject (b). The corresponding values of BSI were:

a. Femoral neck BSI 2.33; Total Hip BSI 1.94; Lumbar spine BSI 3.02

b. Femoral neck BSI 1.13; Total Hip BSI 1.17; Lumbar spine BSI 0.93.



Vertebral fracture assessment. VFA utilized dedicated vertebral fracture assessment software. To confirm VFx among those with scoliosis and disk space osteoarthritis, conventional spinal radiographs (T4-L4) in the lateral and the anteroposterior projections were also performed (287). A single experienced investigator read all images and scored VFx using the Genant semiquantitative method (grade 1, mild; grade 2, moderate; grade 3, severe) (288).

Ethics. The study was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Principles of Good Clinical Practice. The research protocol was approved by local ethics committees and all participants gave informed consent allowing their anonymised information to be used for a data analysis.

4.2.2 Statistical analysis

According to our sample size, there will be a 80% probability to declare as statistically significant at $\alpha = .05$ a standardised difference of 0.5 between groups ("medium-sized" difference according to Cohen)(289). The characteristics of the study participants were reported using descriptive statistics (mean and SD for continuous variables and proportions for categorical variables) by groups defined according to the diagnosis of PHPT or control group. Differences between groups were assessed using the T-test for continuous normally distributed variables and chi-square test for proportions. The associations between FN-BSI, TH-BSI, LS-BSI, LS-BMD, TH-BMD, FN-BMD, calcium, PTH, phosphate, 25 Oh vitamin D, CTX, P1NP and TBS were tested by either Pearson's product-moment correlation or Spearman's correlation as appropriate. Receiver operating characteristic (ROC) curves analysis were created to estimate the best cut-off values of FN-BSI, TH-BSI and LS-BSI for detecting patients with prevalent VFx. Based on these cut-offs, sensitivity (SN), specificity (SP) and overall accuracy of FN-BSI, TH-BSI and LS-BSI were calculated. A p-value of <0.05 was

considered significant. Statistical analysis was performed by SPSS version 26.0 statistical package (SPSS, Inc.).

4.3 Results

Clinical and biochemical characteristics

The study population consisted of 83 adults (44 subjects with PHPT and 39 controls). The mean age of PHPT and controls was 64.5 years (SD 11.9) and 64.7 years (SD 7.08), respectively; 93% of PHPT and 90% of controls were postmenopausal women. There were no differences between groups in terms of age, sex, menopausal age, years after menopause, body mass index (BMI), 25 OH vitamin D and eGFR. The albumin-adjusted serum calcium, ionized calcium, PTH, CTX and P1NP levels were significantly higher in the PHPT group, as expected ($p<0.001$); while phosphorus levels were significantly lower in PHPT compared to controls ($p<0.001$) (Table 8).

Table 8. Baseline characteristics of the study population. Data reported as mean (SD) or n (%)

	Controls, n=39	**PHPT, n=44**	**P- value**
Female, n (%)	35 (90%)	41 (93%)	0.360
Age, years	64.7 (7.08)	64.5 (11.9)	0.763
BMI kg/m^2	26.2(4.7)	27.86 (6.06)	0.225
Menopause age	50.46 (3.36)	50.36(3.97)	0.913
Years of menopause	14 (7.63)	14.29 (9.55)	0.884
25OH VITAMIN D, ng/mL	28.61 (12.8)	32.03(7.62)	0.138
Albumin-adjusted serum calcium, mg/dL	9.49 (0.38)	10.75 (0.43)	**0.001**
Ionized calcium, mmol/L	1.22 (0.05)	1.35 (0.05)	**0.001**

PTH, pg/dL	52.38 (15,36)	133.90 (48.48)	**0.001**
Phosphorus, mg/dL	3.76(0.38)	2.77 (0.47)	**0.001**
Creatinine, mg/dL	0.71(0.1)	0.72 (0.1)	0.540
GFR, ml/min/m^2	86.86 (15.65)	89.93 (24.37)	0.503
CTX, ng/mL	0.33 (0.2)	0.48 (0.27)	**0.010**
P1NP, ng/mL	50.12 (24.14)	71.92 (41.8)	**0.008**
Fractures, n (%)	9 (23)	18 (41)	0.080
TBS	1.28 (0.09)	1.25 (0.09)	0.090
Lumbar T-score adjusted for TBS	-1.95 (1.0)	-2.33 (1.0)	0.080
Femoral Neck BMD, g/cm^2	0.671 (0.07)	0.639 (0.1)	0.100
Femoral Neck T-score	-1.60 (0.67)	-1.90 (0.9)	0.119
Total hip BMD, g/cm^2	0.872 (0.09)	0.802 (0.13)	**0.007**
T-score total hip	-0.6 (0.7)	-1.16 (1.03)	**0.010**
Lumbar spine BMD, g/cm^2	0.904 (0.14)	0.882 (0.20)	0.496
T-score lumbar spine	-1.32 (1.3)	-1.47(1.85)	0.686
1/3 distal radius BMD g/cm^2	0.620 (0.06)	0.565 (0.07)	**0.001**
T-score 1/3 distal radius	-1.33 (0.81)	-2.20 (1.2)	**0.001**

Densitometric features

There were no differences between groups in the femoral neck and lumbar spine BMD, while total hip BMD was lower in the PHPT group than in controls (0.802± 0.13 vs 0.872 ±0.09; $P < 0.05$). The 1/3 distal radius BMD was also lower in PHPT group compared to controls (0.565 ±0,07 vs 0.620 ± 0.06; $P < 0.001$) (Figure 8). There were no differences between groups in TBS and T-score adjusted for TBS (Figure 9). The overall prevalence of morphometric VFs in PHPT and controls was 41% and 23% respectively (p=0.08). Table 8 shows a general overview of the baseline characteristic of our population, with corresponding differences between the two groups.

There was a difference between controls and PHPT in LS-BSI (1.94±0.48 vs 2.20 ± 0.58; p=0.003), FN-BSI (1.40±0.36 vs 1.66±0.39; p=0.003) and TH-BSI (1.24 ± 0.25 vs 1.46±0.3; p=0.001) (Figure 9).

The bivariate correlation analysis showed that TBS was significantly associated with LS-BSI (r=-0.59 p=0.001).

Figure 8. Evaluation of BMD in PHPT and control group. Data are presented as mean (bars) and standard deviation (whiskers). *p<0.05; ** p< 0.001

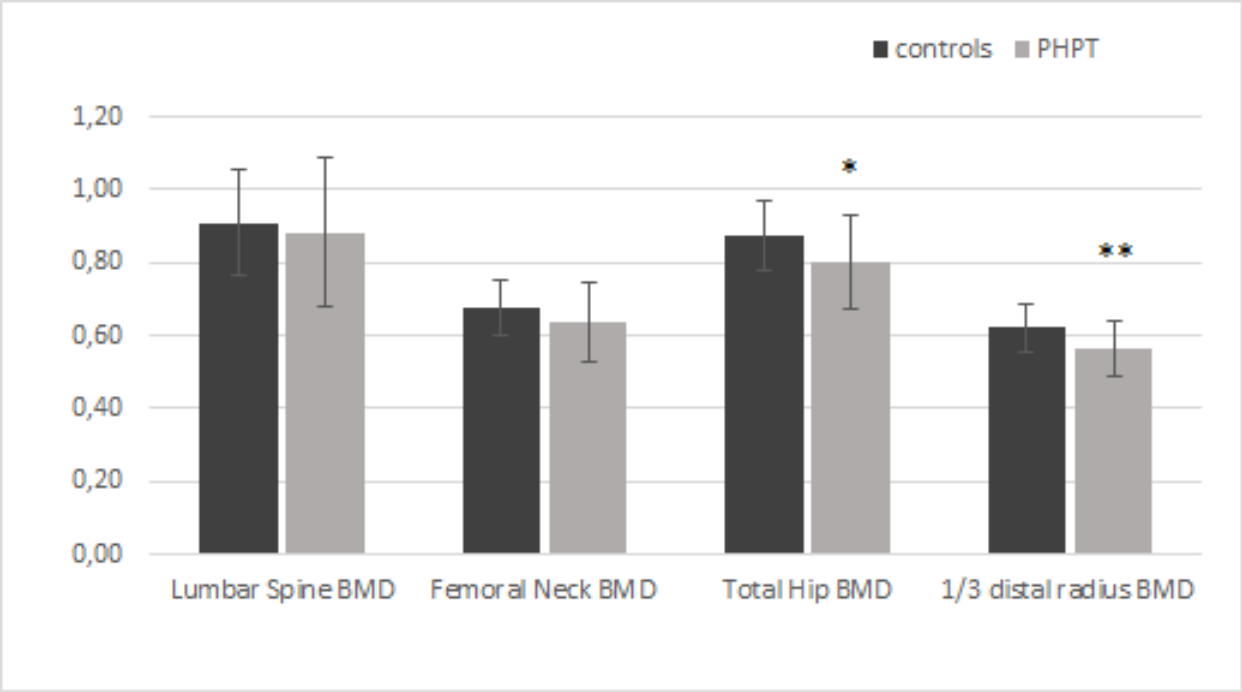
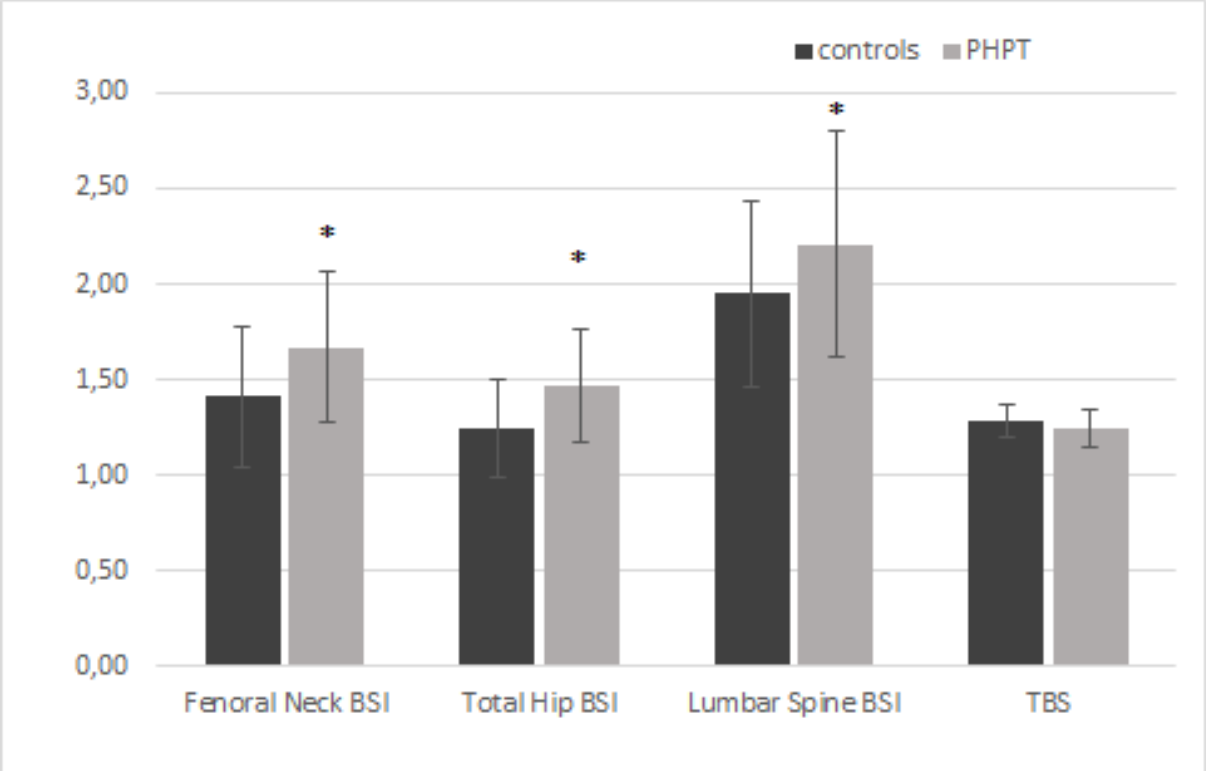


Figure 9. Evaluation of BSI and TBS in PHPT and control group. Data are presented as mean (bars) and standard deviation (Whiskers). *p < 0.01



Accuracy of LS-BSI, FN-BSI AND TH-BSI in detecting subjects with VFx

The ROC showed that LS-BSI has moderate accuracy for detecting VFx (area under the ROC curve 0.68, CI: 0.52-0.85). The cut-off with the best compromise between SN and SP was set at 2.12 (SN 72%, SP 64%, and accuracy 67.4%). The area under the ROC curve for FN-BSI and TH-BSI was 0.64, (CI: 0.42-0.78) and 0.59 (CI: 0.42-0.76), respectively (Table 9).

Table 9. Area under the curve of BSI, TBS and Dexa for detecting PHPT patients with prevalent vertebral fracture.

	AUC	CI (95%)
Lumbar spine-BSI	0.68	0.52-0.85
Femoral Neck-BSI	0.64	0.42-0.78
Total Hip- BSI	0.59	0.42-0.76
TBS	0.35	0.18-0.52
Lumbar T-score adjusted for TBS	0.37	0.20- 0.54
Femoral neck BMD	0.50	0.32-0.67
Total Hip BMD	0.46	0.28-0.63
1/3 distal radius BMD	0.57	0.40-0.75
Lumbar spine BMD	0.37	0.20-0.54

4.4 Discussion

This is the first study to evaluate the BSI in subjects with PHPT. This methodology has shown promise in predicting the risk of vertebral fracture (277). Since DXA of the spine does not predict vertebral or non-vertebral fracture risk in PHPT(161,290–292), other imaging modalities are needed. While TBS has shown promise in several studies (156,157,273,293,294), it represents just one aspect of bone resistance. By applying finite element analysis to the DXA image of defined vertebral or hip regions, the BSI can quantitate bone strength more precisely than TBS. We showed that compared to age-matched controls, patients with PHPT have higher BSI values at the lumbar spine, femoral neck, and total hip. Higher BSI values indicate lower bone strength. Our results are in agreement with HRpQCT, a non-invasive method that is still primarily a research tool (4). By HRpQCT, both cortical and trabecular microstructural elements are compromised (158). The advantage of BSI rests in its facile availability, through the

DXA image of lumbar spine and hip regions, areas that can only be inferred by HRpQCT.

Rendering further uncertain the utility of TBS are the conflicting results from published studies (156,157,273,274,293–295). Regarding our study population, we found a reduction of the mean TBS value in PHPT, but it was not statistically different from age-matched controls. Nevertheless, there was a correlation between LS-BSI and TBS. This is not surprising because both methods are addressing bone quality of the trabecular compartment of bone.

Furthermore, there were no differences between groups in femoral neck BMD or lumbar spine BMD, but BSI was significantly higher (i.e. worse) at all skeletal sites in the PHPT group. The increased BSI at the lumbar spine, a site enriched in trabecular bone, supports the hypothesis that trabecular bone, in addition to cortical bone, is also affected in PHPT.

BSI is based on FEM algorithms, that evaluate the stress/strain conditions of bone when a force is applied, simulating structural deformation. A recent multicenter study performed on BSI analysis of lumbar spine images showed its ability to predict re-fracture in patients with severe osteoporosis (277). The BSI analysis was a significant independent predictor of a subsequent re-fracture. Another study investigated the effect of teriparatide on different DXA-based parameters, including BSI (280). That study showed a positive effect of teriparatide both on TBS and BSI, suggesting that the increase in BMD was accompanied by an increased in bone strength (280). Finally, a study on a cohort of patients with mastocytosis showed a good correlation with a biochemical index that describes the activity of the disease (296). This methodology, thus, has the potential to lend insight into secondary causes of osteoporosis.

If further prospective studies confirm these findings, BSI may well become a useful tool to identify subjects at higher risk of vertebral and non-vertebral fractures.

The study has some limitations. First, cross-sectional studies such as this need to be followed with prospective investigations. Second, as a single site study, we await further single and multi-site studies to add insights into our observations.

Larger prospective studies may well demonstrate the utility of BSI to evaluate bone quality in PHPT.

CHAPTER 5: **RESEARCH PROJECT N°4**

BONE QUALITY, AS MEASURED BY TRABECULAR BONE SCORE IN NORMOCALCAEMIC PRIMARY HYPERPARATHYROIDISM *

**This manuscript is actually under revision.*

5.1 Introduction

Over the past several decades, phenotypic variability in the clinical presentations of primary hyperparathyroidism (PHPT) has become evident (297), due to the widespread use of PTH measurements in the evaluation of those with or suspect to have a metabolic bone disease.

Normocalcemic hyperparathyroidism (NHPT) was first described in 2008, as a condition characterized by levels of albumin-adjusted total serum calcium and ionized calcium consistently within normal limits associated with constantly elevated PTH values after ruling out secondary causes of high PTH (298,299).

Up to now, its true prevalence, natural history and clinical consequences have not been fully clarified. Nevertheless, although few previous studies reported bone impairment in patients with NHPT (217,219,300), the largest recent cross sectional evaluation showed no significant reduction of BMD nor increased number of fragility VFx compared to controls (216).

In both PHPT and NHPT, data on microstructural features by HRpQCT are available. While in PHPT patients, both cortical and trabecular microarchitectural abnormalities have been reported (158), in NHPT subjects HRpQCT showed an impairment in cortical geometric properties with preserved trabecular parameters (221).

However, HRpQCT remains a research tool and other more widely available techniques are required to evaluate in clinical settings bone quality in NPHPT.

TBS is a textural analysis of images acquired by DXA of the lumbar spine (LS) (301). Applied to assess bone microarchitecture, TBS correlates well with standard 3-dimensional (3D) parameters of bone microarchitecture, and appears to be independent of BMD (302,303).

In PHPT, TBS has not consistently stratified fracture risk (156,157,273,274,293,294); in NPHPT, there is essentially only one study (304).

In this report, we present TBS data from a cohort of NHPT patients compared with PHPT patients and control subjects (216).

5.2 Material and methods

5.2.1 Study design and population

Study design and population. Detailed inclusion and exclusion criteria have already been reported elsewhere (216). Briefly, in this study, we consecutively enrolled subjects with PHPT and NHPT at bone outpatient clinics of the following centers: Unit of Endocrinology and Diabetes, Campus Bio-Medico University of Rome; Department of Internal Medicine, "S. M. Goretti" Hospital, Latina; Department of Endocrinology, San Giovanni Addolorata Hospital, Rome and Department of Endocrinology, CTO "A. Alesini" Hospital, Rome.

NHPT was defined as a persistently normal total, albumin-corrected, and ionized serum calcium levels and persistently elevated PTH concentrations (at least 2 different evaluations), after ruling out the secondary causes of hyperparathyroidism such as renal function impairment (eGFR <60 mL/min), hypovitaminosis D (25(OH)D3 <30 ng/mL), daily calcium intake below 700 mg/day and hypercalciuria. All NHPT subjects received supplementation with oral cholecalciferol in order to maintain adequate 25(OH)D3 levels. PHPT was described as elevated or unsuppressed PTH levels and persistently elevated total, albumin-corrected, or ionized serum calcium levels (at least 2 different evaluations).

We excluded subjects with any other condition that can affect bone and calcium metabolism, malabsorption diseases, administration of drugs affecting bone and calcium metabolism, history of possible traumatic vertebral fractures, metabolic bone diseases such as Paget disease and osteogenesis imperfecta.

Control subjects without any abnormalities of calcium, phosphate and PTH levels were consecutively recruited based on the above-mentioned exclusion criteria from the outpatient clinic of endocrinology at Campus Bio Medico University of Rome, where they were referred for unrelated diseases (thyroid nodules with euthyroidism). Before enrollment, all controls had never performed a BMD evaluation. In all control subjects with low BMD, we ruled out secondary causes of osteoporosis in accordance with the current edition of Italian guidelines (305).

Clinical and biochemical evaluation. We performed a physical examination and reviewed the previous medical records in order to evaluate the clinical profile of the whole study population.

In the morning (from 8:00 to 8:30 AM) fasting blood samples were obtained. Serum total calcium (normal, 8.4–10.2 mg/dL), albumin (normal 3.2-4.6 g/dl), serum phosphate (normal, 2.3-4.7 mg/dL), creatinine (normal 0.55-1.2 mg/dl) and 25(OH) D (normal, 30-100 ng/mL) were measured using automated methods. Calcium values were corrected for albumin concentration using the formula: corrected calcium (mg/dL) = measured total calcium (mg/dL) + 0.8 (4.0 – serum albumin [g/dL]). Ionized serum calcium (normal, 1.13–1.32 mmol/L) was measured by potentiometric method on the GEM PREMIER 4000 analyzers (Werfen, Le Pré-Saint-Gervais, France). Intact PTH (normal, 14-72 pg/ml) was measured by an immunochemiluminometric assay using the automatic analyzer Modular E170 (Roche Diagnostics, Indianapolis, Ind, USA) in the Laboratory at Campus Bio-Medico University of Rome. 24-h urinary calcium was assessed by calorimetry. eGFR was calculated using the MDRD equation. Serum levels of CTX were assayed by the β-CrossLaps (ECLIA; β-CrossLaps/Serum, Roche Diagnostics, Basel, Switzerland), which uses two monoclonal antibodies against

β-cross-linked CTX according to the manufacturer's protocol. Serum levels of P1NP were analysed by sensitive electrochemiluminescent detection technology and was formatted for the Cobas Total P1NP (Roche Diagnostics) automated analyser.

Dual-energy X-ray absorptiometry. BMD by DXA was measured at the LS (L1–L4), total hip (TH), femoral neck (FN), and non-dominant forearm (one-third distal radius-Radial) using a dual-energy X-ray absorptiometer (Hologic Discovery QDR Instrument, MA, USA). Data was reported as absolute bone density, Z-score (SD values from the mean for gender, ethnicity and age-matched healthy population) and T-scores (SD values from the mean for a sex- and young reference population). All scans were performed according to the International Society for Clinical Densitometry (ISCD) guidelines (281). Fractured vertebrae and vertebrae with structural changes were excluded from the analysis (T-score difference with the adjacent vertebra > 1.0).

Vertebral fracture assessment (VFA). VFx were evaluated by DXA scanning of the spine for VFA by quantitative morphometry (QM). In order to confirm these findings in presence of scoliosis and disc space osteoarthritis (287), conventional spinal radiographs (T4–L4) in lateral and antero-posterior projection was performed. A single experienced investigator read all images and scored VFx using the Genant semiquantitative method (grade 1 – mild, grade 2 - moderate, grade 3 - severe) (288).

Trabecular bone score. TBS was automatically derived from the same region of LS DXA by a dedicated software (Insight TBS, Medimaps Groupe, Geneve, Switzerland). TBS was calculated as the mean value of the individual determinations for LS vertebrae L1–L4. Vertebrae excluded for BMD measurement were also excluded for TBS evaluation at LS. Fracture-resistant microarchitecture is reflected by higher TBS instead lower values may reflect frail microarchitecture (302). We defined low TBS as TBS <1.200(306).

Ethics. The study was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Principles of Good Clinical Practice. The research protocol was approved by Local Ethics Committees and all participants

gave informed consent allowing their anonymized information to be used for a data analysis.

5.2.2 Statistical analysis

The characteristics of the study participants were reported using mean and standard deviation for continuous variables and proportions for categorical variables, by groups defined according to the diagnosis of NHPT, PHPT or control group. Differences between groups were analyzed using analysis of variance for continuous variables and $\chi 2$ test for proportions, and a Tukey test for the post-hoc comparisons between groups was performed.

Receiver operating characteristic (ROC) curves analysis were created to estimate the best cut-off values of TBS, LS Z-score, LS Z-score adjusted for TBS (LS Z-score*TBS), LS T-score adjusted for TBS (LS T-score*TBS), Radial Z-score and FN Z-score for detecting patients with overall VFx or moderate-severe VFx. On the basis of these cut-offs, sensitivity (SN), specificity (SP) and overall accuracy of these parameters were calculated. A further analysis was performed to compare the diagnostic accuracy of FN Z-score alone with that of the combination FN Z-score plus TBS using the c statistic.

5.3 Results

Baseline characteristics

The study population consisted of 127 adults (47 subjects with NHPT, 41 with PHPT and 39 controls). Table 10 summarizes key patient demographics and baseline characteristics. The mean age of the study population was 64.1 years (SD 9.6); 91% female (postmenopausal women).

In particular, patients with NHPT presented higher Radial BMD compared with PHPT (0.605, SD 0.080 vs 0.563, SD 0.078, P = .031). The prevalence of VFs was 28% in NHPT group, 23% in controls and 60% in PHPT group.

Table 10. General characteristics of the population. Data reported as mean (Standard Deviation) or n (%)

	Controls **N: 39**	**NHPT** **N: 47**	**PHPT** **N: 41**
Age (years)	64.7 (7)	63.8 (9.3)	63.9 (12)
Female (%)	90	91	93
BMI (kg/m^2)	26.2 (4.7)	26.7 (4.9)	27.4 (5.3)
Time from menopause (years)	14 (7.6)	13.7 (9.8)	14 (9.6)
eGFR (ml/min/1.73 m^2)	86.86 (15.65)	83.44 (23.68)	92.11 (24.46)
Serum calcium concentration (mg/dl)	9.5 (0.4)	9.4 (0.4)$^{++}$	10.8 (0.4)**
Serum calcium ion concentration (mmol/l)	1.22 (0.05)	1.19 (0.05)$^{++*}$	1.35 (0.05)**
24-h urine Calcium (mg/24h)	192.3 (76)	196.1 (49.2)$^{++}$	293.5 (146.3)**
Serum phosphate concentration (mg/dl)	3.8 (0.4)	3.2 (0.5)$^{**++}$	2.8 (0.5)**
Serum PTH concentration (pg/ml)	52.4 (15.4)	126.8 (29.5)**	139.1 (49.7)**

25-OH Vitamin D concentration (ng/ml)	28.6 (12.8)	36.7 (6.6)**	31.1 (7.8)*
Calcium*Phosphorus	35.66 (3.84)	29.55 (4.42)**	29.78 (5.26)**
Serum CTX concentration (ng/ml)	0.33 (0.21)	0.37 (0.18)	0.49 (0.27)*
Serum P1NP concentration (ng/ml)	50.12 (24.14)	61.33 (25.41)	73.09 (42.09)*
TBS	1.283 (0.09)	1.290 (0.127)	1.244 (0.103)
TBS<1.2 (%)	15.4	23.4	26.8
T-score*TBS	-1.95 (1.00)	-1.93 (1.30)	-2.37 (1.05)
Z-score*TBS	0.07 (0.93)	-0.04 (1.12)	-0.48 (1.06)*
LS BMD (g/cm^2)	0.904 (0.149)	0.893 (0.186)	0.880 (0.184)
LS T-score	-1.3 (1.3)	-1.4 (1.7)	-1.5 (1.6)
LS Z-score	0.32 (1.44)	0.20 (1.70)	0.11 (1.81)
FN BMD (g/cm^2)	0.671 (0.075)	0.659 (0.108)	0.633 (0.107)
FN T-score	-1.6 (0.7)	-1.8 (0.9)	-2 (1)
FN Z-score	-0.17 (0.69)	-0.29 (0.94)	-0.49 (0.89)
TH BMD (g/cm^2)	0.872 (0.097)	0.819 (0.125)	0.795 (0.126)*
TH T-score	-0.6 (0.7)	-1.1 (0.9)	-1.2 (1)*
TH Z-score	0.52 (0.79)	0.08 (0.96)	-0.07 (1.05)*
Radial BMD (g/cm^2)	0.620 (0.065)	0.605 (0.080)+	0.563 (0.078)*
Radial T-score	-1.3 (0.8)	-1.6 (1.2)+	-2.3 (1.3)**

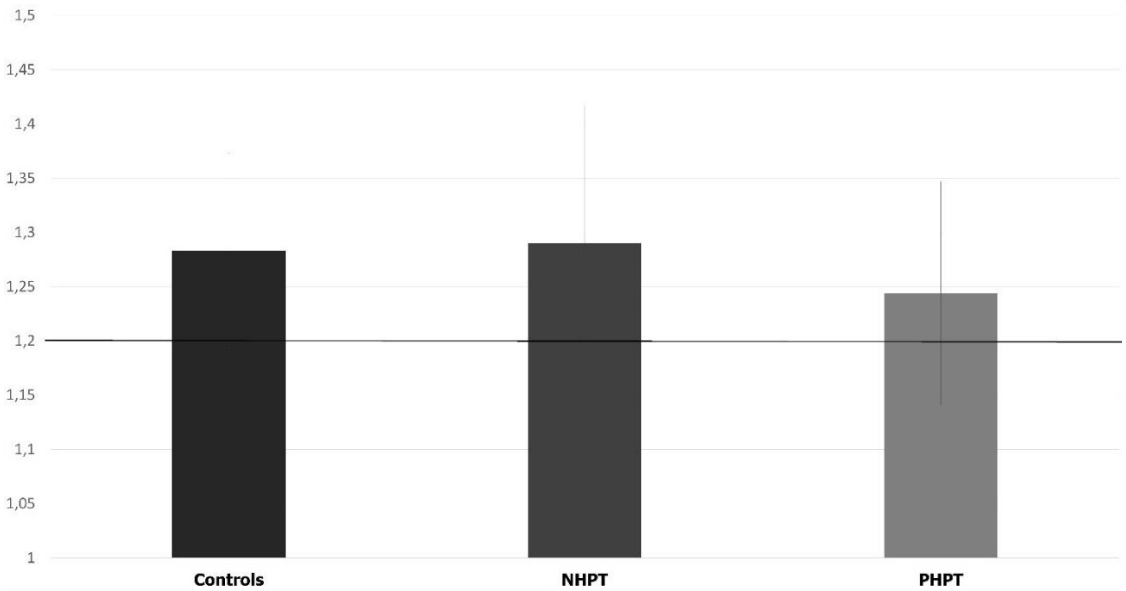
Radial Z-score	0.33 (0.87)	0.08 (0.98)$^{+}$	-0.53 (1.15)**
Vertebral fractures (%)	23	28	60*
Renal lithiasis (%)	3	13	10

**P<0.001 vs control group; *P<0.05 vs control group; ++P<0.001 vs PHPT; +P<0.05 vs PHPT

Trabecular bone score

We did not find any significant difference in terms of TBS and LS T-score*TBS between NHPT participants, PHPT group and controls (Figure 10; Table 10). The same results were observed considering LS T-score*TBS. We found a lower LS Z-score*TBS in PHPT participants when compared with controls (-0.48 ± 1.06 vs 0.07 ± 0.93, p: 0.017) (Table 10). Moreover, the prevalence of low TBS was 23.4% in NHPT, 26.8% in PHPT and 15.4% in controls, without statistically differences between groups. Furthermore, we found that subjects with low TBS had higher BMI compared with those with TBS>1.200 in both NHPT (30.42 kg/m2 ± 5.73 vs 25.75 kg/m2 ± 4.19, p: 0.034) and PHPT (32.28 kg/m2 ± 4.59 vs 25.82 kg/m2 ± 4.49, p<0.001) groups.

Figure 10. TBS in controls, NHPT and PHPT participants



Accuracy of trabecular bone score in detecting subjects with vertebral fractures

The ROC analyses have been performed to assess the best threshold values of TBS, LS Z-score, LS Z-score*TBS, LS T-score*TBS, Radial Z-score, FN Z-score for detecting all VFx in NHPT group. The results showed that all these parameters are not able to detect overall VFx in NHPT group (Table 11).

Table 11. Diagnostic performance of DXA-derived parameters and TBS in detecting overall VFx in NHPT participants

	AUC (95% CI)	Threshold	Specificity	Sensitivity	Accuracy
TBS	0.515 (0.327-0.703)	1.254	0.676	0.538	0.638
LS Z-score*TBS	0.45 (0.253-0.648)	-0.15	0.559	0.538	0.553
LS T-score*TBS	0.509 (0.321-0.698)	-2.45	0.706	0.462	0.638
LS Z-score	0.457 (0.269-0.645)	0.25	0.485	0.615	0.522
Radial Z-score	0.489 (0.289-0.688)	0.2	0.606	0.5	0.578
FN Z-score	0.678 (0.499-0.858)	-0.05	0.758	0.692	0.739

Furthermore, LS T-score*TBS demonstrated a moderate accuracy for identifying moderate-severe VFx in subjects with NHPT (Table 12).

Table 12. Diagnostic performance of DXA-derived parameters and TBS in detecting moderate-severe VFx in NHPT participants

	AUC (95% CI)	Threshold	Specificity	Sensitivity	Accuracy
TBS	0.714 (0.461-0.967)	1.304	0.667	0.8	0.681
LS Z-score*TBS	0.81 (0.62-0.996)	0.55	0.833	0.8	0.83
LS T-score*TBS	0.719 (0.464-0.952)	-0.85	0.857	0.6	0.83
LS Z-score	0.705 (0.497-0.913)	-0.05	0.463	1	0.522
Radial Z-score	0.71 (0.54-0.88)	0.2	0.634	1	0.667
FN Z-score	0.624 (0.364-0.885)	-0.95	0.293	1	0.37

Regarding PHPT group, TBS was not able to detect overall VFx (Threshold 1.292, AUC 0.52 95%CI 0.336-0.718) and the same findings have been confirmed also for the identification of moderate-severe VFx (data not shown).

The combination of FN Z-score with TBS does not improve the ability of overall VFs detection compared to FN Z-score alone in both NHPT (c statistic 0.683 vs 0.678, P=0.715) and PHPT (c statistic 0.569 vs 0.548, P=0,830) population.

5.4 Discussion

This is the first study that systematically evaluated the TBS, a surrogate marker of bone quality, in a large cohort of well-defined subjects with NHPT. We showed that compared to controls and PHPT, NHPT population did not have statistically different TBS values. Moreover, TBS tool may increase the accuracy of DXA in individuating only moderate-severe VFx, while it seems not to be able to capture patients with mild VFx.

It has been well demonstrated that bone strength and fracture risk are influenced also by other factors than bone mass (307), and this is one of the reasons why BMD assessed by DXA is not able to adequately predict the increased risk of fractures in patients with PHPT (160,161).

While some studies investigated bone quality by HRpQCT in PHPT subjects (158,308), only one investigation used this tool in NHPT patients. In that study, an impairment of cortical bone microstructure was reported, but the inclusion of subjects with 25(OH)D3 levels <30 ng/ml might have influenced that finding (221).

However, while HRpQCT still remains a research tool, TBS is a DXA-derived index of bone microarchitecture that is simple and easy to perform in clinical practice (303).

Indeed, TBS has been demonstrated to be an accurate tool in predicting fracture risk in many clinical settings of secondary osteoporosis (309,310). In particular, in subjects with diabetes, TBS captured fracture risk and skeletal deterioration more accurately than BMD (311,312). Furthermore, TBS has greater discriminative power than BMD for bone quality deterioration and fracture risk assessment in subjects with

glucocorticoid-induced osteoporosis (313), remarkably also in those with only a mild cortisol hypersecretion (314).

This latter finding has rendered the TBS determination in NHPT, which is considered by some authors a possible mild form of PHPT (134,223,315) as an interesting area of investigation.

However, the utility of TBS in predicting fragility fractures in patients with PHPT is still uncertain (156,157,273,274,293,294). In particular, some authors (157,273,293,294) showed a significant reduction of TBS in PHPT subjects that seems to be associated with VFx after adjustment for confounding factors. Likewise, Silva et al. (156) reported significant positive correlations between TBS and measurements of trabecular microarchitecture assessed by HRpQCT, suggesting that low TBS may also indicate impaired bone strength. Conversely, in the largest study published to date, Grigorie et al. (274) showed that even if PHPT patients had mean TBS values in the partially degraded range, TBS was not independently associated with fractures. In our cohort, PHPT showed a lower LS Z-score*TBS compared to controls even if it was not able to detect VFx in this clinical setting.

Moreover, Tay et al. did not reveal any significantly impaired values of TBS in nonobese PHPT subjects (295). We founded that both NHPT and PHPT with fully degraded skeletal microarchitecture, as measured by TBS, had higher BMI than those with TBS>1.200. These findings suggest the detrimental effect of obesity on bone quality, as previously reported in PHPT (295).

Longitudinal studies using TBS in PHPT are limited, and data regarding TBS changes after parathyroidectomy are largely contradictory (157,295).

To date, the only study investigating TBS in NHPT subjects (304) was not able to demonstrate differences in TBS between NHPT and asymptomatic PHPT patients. The small sample size, the inability to rule out all causes of secondary hyperparathyroidism and the lack of fractures evaluation rendered the results of this study incomplete.

In accordance with that previous study, we did not find significant differences in terms of TBS or Z-score*TBS between NHPT and the other groups. Furthermore, this tool did not provide an additional advantage in detecting overall VFx compared to BMD. The ability to predict overall VFx remains poor even when combining the TBS with FN-Z-score, in both in the NHPT and PHPT groups. LS Z-score*TBS has an acceptable accuracy (83%) in detecting moderate-severe VFx in NHPT group. This may be in line with the fact that TBS seems to reflect moderate-severe deteriorations of bone quality while it is not able to detect small reductions in bone quality (316). Therefore, it is not surprising that LS Z-score*TBS could be usefully used for detecting patients with moderate-severe VFx, that represent a moderate-severe reduction of bone quality, rather that mild VFx, that account for a slight bone quality reduction (317).

We already showed that in NHPT bone density is not remarkably reduced and is similar to that the bone phenotype of healthy control subjects (216). This further data of unimpaired bone quality assessed by TBS in NHPT group support the hypothesis that this condition may represent a very early stage of classic PHPT (134,223,315). However, the finding that in NHPT the TBS determination could increase the accuracy in detecting moderate-severe VFx encourages further studies along these lines.

Our evaluation has particular strengths: we have studied one of the largest cohorts of NHPT and biochemical and radiological assessments were conducted in a single center. Our study is limited by its cross-sectional design and the lack of use of a third generation PTH assay.

Notwithstanding the afore-mentioned limitations, the results of the present study suggest that NHPT patients do not show significant impairment of bone microarchitecture as assessed by TBS. In NHPT, TBS does not appear to identify patients with mild VFx. Rather, it might offer advantages in identifying those with moderate-severe VFx.

IMPACT OF MY RESEARCH PROJECT AND FUTURE PERSPECTIVES

1) Cardiovascular autonomic neuropathy as a new complication of postsurgical chronic hypoparathyroidism.

There are few reports of peripheral neuropathy associated with hypoparathyroidism. Moreover, patients with post-surgical hypoparathyroidism seem to show a two-fold increased risk of death after adjusting for age, sex, co-morbidities at the time of thyroidectomy and thyrotoxicosis as the indication for thyroidectomy. The cause of this increased mortality in hypoparathyroidism subjects is not clear but might be linked to lower PTH levels. For the first time, we showed that chronic postsurgical hypoparathyroidism is associated with cardiovascular autonomic neuropathy and that hypocalcemia seems to affect its severity. Cardiovascular autonomic neuropathy is defined as the impairment of the autonomic control of the cardiovascular system and different approaches have been used to diagnose this condition in practice or research. We performed the cardiovascular autonomic reflex tests to investigate the integrity of the autonomic nervous system because they are sensitive, specific, reproducible, safe, standardized and eminent guidelines recommended their use as the gold standard for clinical autonomic testing.

Future perspectives

- According to the previous findings, we designed a trial aimed to evaluate the impact of the chronic lack of PTH in subjects with post-surgical hypoparathyroidism on central and autonomic nervous system and on ocular surfaces since it is the most innervating tissue in the human body and easily reachable. During the study, all patients will perform also cardiovascular autonomic neuropathy evaluation only with lying to standing test (due to COVID-19 pandemic breath tests should not be done) and assessing heart rate variability. We are collecting the data from patients.

- According to the previous findings, we designed a trial aimed to evaluate the impact

of the chronic lack of PTH in subjects with post-surgical hypoparathyroidism on platelet and endothelial functions (measured by flow-mediated dilation) in comparison with subjects affected by hyperparathyroidism and controls. We are collecting the data from patients.

2) Cardiovascular autonomic neuropathy as a cause of fatigue in chronic hypoparathyroidism.

Patients with hypoparathyroidism, and particularly patients with postsurgical hypoparathyroidism, often experience symptoms suggestive of a poor quality of life. Furthermore, patients with long-standing postsurgical hypoparathyroidism can be a good model for studying quality of life because they were used to living with normal serum calcium before surgery. The need to take large amounts of calcium and calcitriol made the patient feel dependent on drugs and without the benefit of achieving an acceptable quality of life. The most common symptoms are fatigue, muscle cramps, paraesthesia, pain, poor memory, a lack of concentration, depression, and anxiety. This is the first study showing that cardiovascular autonomic neuropathy might be associated with fatigue in subjects with chronic postsurgical hypoparathyroidism. We can speculate that fatigue in hypoparathyroidism patients is a result of the inability of the heart to adjust its function to the physical activity level of the subject.

Future perspectives

- We would like to design a trial aimed to evaluate the quality of life and fatigue in correlation with the cardiovascular autonomic neuropathy before and after treatment with PTH analogs. PTH replacement therapy already demonstrated an improvement of quality of life in this condition. Up to now, no trial evaluated if the improvement of quality of life may by be due to the improvement of cardiovascular autonomic neuropathy.

3) Dxa-based bone strain index: a new tool to evaluate bone quality in primary hyperparathyroidism.

Although increased fractures risk at both vertebral and non-vertebral sites are well established clinically, bone mineral density is typically reduced at the distal 1/3 forearm, a primarily cortical bone site, with relative preservation of the lumbar spine, a predominantly trabecular site. In order to evaluate bone quality in the clinical setting, more readily available indices are needed. Until recently, trabecular bone score has been the main DXA-based tool measuring bone quality in PHPT. Questions remain, however, as to whether vertebral fracture can be easily detected in this clinical setting. Bone strain index is a new DXA-derived skeletal parameter of deformation that is based on Finite Element Method. This is the first study to evaluate the BSI in subjects with PHPT and this technology has shown promise in predicting the risk of vertebral fracture. Our results are in agreement with high resolution peripheral quantitative computed tomography, a non-invasive method that is still primarily a research tool. If further prospective studies confirm these findings, Bone strain index may well become a useful tool to identify subjects at higher risk of vertebral and non-vertebral fractures.

Future perspectives

- We would like to design a prospective trial aimed to evaluate the bone quality assessed by bone strain index in subjects affected by PHPT before and after parathyroidectomy. It would be interesting to investigate also the cardiovascular autonomic neuropathy before and after parathyroidectomy and to correlate this condition with patients' bone status.

- We would like to design a trial aimed to evaluate the bone quality assessed by bone strain index in other secondary causes of osteoporosis such as glucocorticoid-induced osteoporosis.

4) Bone quality, as measured by trabecular bone score in normocalcaemic primary hyperparathyroidism.

Although the Fourth International workshop for the management of asymptomatic PHPT tries to clarify its diagnostic criteria and bone impairment of subjects with NHPT, no trials have evaluated the accuracy of TBS in identifying fractures in this kind of population. This is the first study that systematically evaluated the TBS, a surrogate marker of bone quality, in a large cohort of well-defined subjects with NHPT. TBS is a DXA-derived index of bone microarchitecture that is simple and easy to perform in clinical practice. We showed that compared to controls and PHPT, NHPT population did not have statistically different TBS values. Moreover, TBS tool may increase the accuracy of DXA in individuating only moderate-severe VFx, while it seems not to be able to capture patients with mild VFx.

However, the use of this tool in NHPT demonstrating that the TBS determination could increase the accuracy in detecting moderate-severe VFx encourages further studies along these lines.

Future perspectives

-We designed a new trial aimed to evaluate the bone material properties assessed by microindentation technique in NHPT compared to PHPT and healthy subjects.

-We would like to design a trial aimed to evaluate the bone quality assessed by bone strain index in NHPT compared to PHPT and healthy subjects. Bone strain index is a new software able to detect fracture risk and never used up to now in this condition.

PUBLICATIONS AND PAPERS DERIVED FROM THIS WORK

Tabacco G*, **Naciu AM***, Maggi D, Santonati A, Pedone C, Cesareo R, Bosco D, Gaspa G, Napoli N, Pozzilli P, Manfrini S, Palermo A. ***Cardiovascular Autonomic Neuropathy as a New Complication of Postsurgical Chronic Hypoparathyroidism.*** *J Bone Miner Res. 2019 Mar;34(3):475-481. *these authors contributed equally.*

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