Background: Normocalcemic primary hyperparathyroidism (PHPT-N) is a condition that may have similar long-term implications to primary hyperparathyroidism (PHPT); however, differential diagnosis and treatment for parathyroid disorders are not clearly defined. We investigated the effect of an oral peptone and an oral calcium load on calcium-regulating hormones in PHPT-N compared with PHPT and healthy controls to provide a new potential diagnostic tool.

Methods: We evaluated serum gastrin, PTH, ionized calcium, and phosphate responses to oral calcium (1 g) and peptone (10 g) load in 22 PHPT and 20 PHPT-N patients matched for PTH serum values. Moreover, 30 healthy subjects were enrolled as controls. In 12 patients for each group, we also performed the oral peptone test adding aluminum hydroxide (AH) to suppress phosphate absorption.

Results: In PHPT patients, PTH increased significantly 30 min after the oral peptone load, while no significant increase was found in PHPT-N and controls. After oral calcium load, PTH remained stable in PHPT while it decreased dramatically in PHPT-N patients, and ionized calcium increased significantly in each of the three groups. Peptones plus AH induced a blunted PTH increase in the three groups.

Conclusions: Considering the marked difference in PTH response elicited by peptones in PHPT compared with PHPT-N, we suggest that the oral peptone test could be added to the diagnostic evaluation of PHPT patients. In case of absent response to peptones, patients should have their serum calcium levels assessed twice a year in accordance with recent guidelines.

Introduction

Primary hyperparathyroidism (PHPT) is a condition in which, in the absence of a known or recognized stimulus, one or more of the four parathyroid glands secrete excess parathyroid hormone (PTH), resulting in hypercalcemia (1, 2). This condition leads to different skeletal and metabolic complications like osteoporosis, nephrocalcinosis, and nephrolithiasis (3, 4). For this reason, PHPT patients in the presence of these complications are candidates for surgery (5). Similarly, recent guidelines outlined some criteria for parathyroidectomy in those PHPT patients without skeletal or renal complications (asymptomatic PHPT patients) (6, 7, 8). The same guidelines, however, state that another clinical entity, namely normocalcemic primary hyperparathyroidism (PHPT-N), characterized by persistently normal calcium concentrations with persistently abnormal PTH levels, exists in the absence of a recognizable underlying cause of elevated PTH serum levels (5). Despite the fact that PHPT-N may also have similar long-term implications as other forms of PHPT (both symptomatic and nonsymptomatic) (9), at the present time, no clinical or surgical indications are present for this condition (5). Due to the existence of slightly different clinical presentations, it is challenging to perform a correct differential diagnosis of PHPT especially in the case of normal calcium levels. In the past, dynamic studies with oral calcium load have been proposed as a completion of diagnosis of PHPT in patients with symptoms who have minimal, intermittent, or no elevation of the levels of total calcium and/or intact PTH (10, 11, 12, 13, 14). However, these studies gave contrasting results and, at the present time, no dynamic/stimulative test has been specifically performed in PHPT-N.
It has been previously demonstrated that L-amino acids are able to modulate calcium-sensing receptor (CASR) through an allosteric pathway, suggesting an interaction between calcium and protein metabolism at gastrointestinal level (15, 16, 17). Following this hypothesis, our group in the last few years investigated the effects of an oral test with peptones rich in L-amino acids and phosphate on calcium-regulating hormones in different pathological conditions (PHPT, hypercalcemia, and bariatric surgery) to elucidate the role of CASR and phosphate in vivo (18, 19, 20, 21).

The question whether PHPT-N patients have a true disorder, one that will cause them the same renal and skeletal complications as PHPT, remains unsolved. For all these reasons, current guidelines recommend that PHPT-N frequency, natural history, and optimal management remain key topics for further evaluation and research in the next years (5).

In light of these recommendations, we investigated the effect of an oral peptone load rich in phosphate compared with an oral calcium load on calcium-regulating hormone handling in patients with PHPT-N, PHPT, and a control group of healthy subjects. The objective of our study is to provide a new potential diagnostic tool in PHPT differential diagnosis and to have a deeper insight into physiopathological mechanisms regulating both PHPT-N and PHPT.

Materials and methods

Subjects

The study patients were selected from those attending the Endocrinology Unit of Luigi Sacco Hospital, Milan, Italy, from September 2010 to December 2011. Thirty PHPT patients were evaluated before surgery, which resulted in a histopathological diagnosis of parathyroid adenoma in 22 patients and parathyroid hyperplasia in eight patients. The inclusion criteria for PHPT patients were ionized calcium serum levels >1.36 mmol/l and high serum levels of intact PTH. The exclusion criteria were familial PHPT, PHPT related to MEN1 or MEN2, potential causes of secondary hyperparathyroidism like renal insufficiency or vitamin D insufficiency, liver diseases, malabsorption, hypercalcemia, Paget’s disease, and thiazide diuretic or lithium use. To obtain a more homogeneous population, only patients with a single adenoma diagnosed after surgery were considered eligible for the study and included in the analysis; moreover, among these 22 patients, all became eucalcemic after surgery. Twenty PHPT-N patients were enrolled in the study according to the following inclusion criteria: presence of high serum levels of intact PTH and serum ionized calcium levels in the high normal range (between 1.27 and 1.30 mmol/l) at two different evaluations at least 2 months apart. The exclusion criteria were the same as for PHPT patients. Moreover, in order to have comparable data, we enrolled only PHPT and PHPT-N patients with PTH serum levels ranging from 80 to 120 pg/ml. After enrollment, PHPT-N patients underwent a parathyroid PET/CT scan that resulted in a diagnosis of single parathyroid adenoma in 50% of patients and no pathological findings in the others. Thirty healthy subjects of both gender were enrolled as controls. Controls and patients had normal renal function, as evaluated by estimated glomerular filtration rate, and 25(OH) vitamin D serum levels ≥ 30 ng/ml (75–80 nmol/l) (22); moreover, 80% of patients and 75% of controls were supplemented with cholecalciferol 8000 IU weekly. All controls had ionized calcium serum levels and 1–84 PTH levels in the normal range. All study participants gave their informed consent, and the institutional review board approved the study, which was conducted in accordance with the guidelines of the Declaration of Helsinki for human research.

Oral test administration

Oral calcium test (1 g calcium gluconate; Calcium Sandoz fortissimo, administered with a slight breakfast) and peptone-meal test (10 g Liebig meat extract diluted in 250 ml of 0.9% saline + 50 ml plain water) were performed as described previously (18, 19). PHPT patients performed the oral tests before surgery. In 12 patients, we repeated the oral peptone test after 1 month, adding cold aluminum hydroxide (AH – Maalox©, Sanofi-Aventis, Milan, Italy, 40 ml), a nonabsorbable phosphate analog that blocks phosphate absorption immediately after peptone administration (23). All the tests were well tolerated and no side effects were observed. Before all testing, all subjects underwent 15 days of low-calcium diet.

Analytical methods

All blood and urine tests were performed at the same hospital. Serum levels of intact PTH, ionized calcium, gastrin, and phosphate were evaluated 15 min before administration of the peptone meal, at oral load administration, and 15, 30, 45, 60, 90, and 120 min after the oral load. Serum immuno reactive 1–84 PTH was evaluated with a Nichols kit (Intact PTH assay, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), plasma ionized calcium was measured using the Nova Stat Profile M, and Gastrin was assessed as described previously (24). The normal values and coefficients of variation for each assay are as follows: ionized calcium, 1.15–1.30 mM/l, 6%; phosphate, 3.0–4.5 mg/dl, 6%; intact PTH, 10–65 pg/ml, 7%; and gastrin, 10–100 pg/ml, 7%. Blood and urinary calcium, phosphate, magnesium, and creatinine were measured with the Technicon SMA-12/60 AutoAnalyzer.

Statistical analyses were performed using GraphPad version 4.0 (GraphPad Software, San Diego, CA, USA).
The differences between parameters obtained during the oral loading tests were calculated with ANOVA for repeated measurements. Differences between single variables in different groups were evaluated with the Mann–Whitney U test. A type I error level of 0.05 was chosen. The Bonferroni correction for multiple comparisons was applied considering four variables, which resulted in a new α-error level of 0.012. P values < 0.01 were considered statistically significant.

Results

The demographic characteristics of the subjects enrolled in the study are summarized in Table 1. The mean ± S.E.M. of the responses to oral peptones in PHPT-N patients, PHPT patients, and control subjects is shown in Fig. 1. Serum PTH levels at baseline were similar in PHPT-N and PHPT patients but statistically different compared with controls, while phosphate and ionized calcium were statistically different between the three groups. In subjects with PHPT, there was a significant increase in serum PTH levels 30, 45, 60, 90, and 120 min after the oral peptone load. PTH levels were statistically different between PHPT-N and PHPT patients only after 60 min (Fig. 1). Ionized calcium decreased significantly at 90 min from oral peptone administration in PHPT patients and after 120 min in healthy controls and PHPT-N patients. A significant increase in serum phosphate levels after oral peptones was observed in PHPT-N and PHPT patients but not in healthy controls. In PHPT patients, we observed blunted responses of gastrin after the oral peptone load with an increase about half of that observed in PHPT-N patients and PHPT patients only after 60 min (Fig. 1). Ionized calcium decreased significantly at 90 min from oral peptone administration in PHPT patients and after 120 min in healthy controls and PHPT-N patients. A significant increase in serum phosphate levels after oral peptones was observed in PHPT-N and PHPT patients but not in healthy controls. In PHPT patients, we observed blunted responses of gastrin after the oral peptone load with an increase about half of that observed in PHPT-N patients and healthy controls (Fig. 2). An oral peptone load with added AH led to a gradual but smaller increase in plasma phosphorus levels than an oral peptone load alone in all groups (Fig. 3). Peptones plus AH induced a blunted increase in serum PTH levels in PHPT and PHPT-N patients compared with an oral peptone load alone. Moreover, unlike peptone load alone, this blunted PTH response was not statistically significant. In PHPT patients, the ionized calcium decrease was smaller after peptones plus AH than after peptones alone, while in the other two groups ionized calcium was unaltered. In all groups, gastrin showed similar trends after an oral peptone load plus AH or oral peptones alone. The mean ± S.E.M. of the responses to oral calcium load in PHPT-N patients, PHPT patients, and control subjects is shown in Figs 1 and 2. After oral calcium load, PTH remained stable in PHPT while decreased dramatically in PHPT-N patients after 15 min and in controls after 60 min. Ionized calcium increased significantly in each of the three groups, while gastrin increased significantly only in healthy controls and PHPT-N. After oral calcium load, serum phosphate increased only in PHPT-N and controls after 120 min. Magnesium levels did not change after both peptone and calcium load in the three groups (data not shown). All patients and controls had normal serum 25(OH) vitamin D levels at baseline and the levels were not statistically different between groups (Table 1).

Table 1 Demographic characteristics of PHPT patients, PHPT-N patients, and healthy controls. Data are presented as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PHPT</th>
<th>PHPT-N</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>58.3±5.1</td>
<td>55.3±6.2</td>
<td>62.6±4.7</td>
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<td>Gender (M/F)</td>
<td>12/10</td>
<td>7/13</td>
<td>12/18</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.4±2.4</td>
<td>24.6±1.8</td>
<td>25.5±1.3</td>
</tr>
<tr>
<td>25(OH)D serum levels (ng/ml)</td>
<td>38.4±5.87</td>
<td>36.7±6.78</td>
<td>43.2±7.14</td>
</tr>
<tr>
<td>25(OH)D serum levels (min–max)</td>
<td>30–43</td>
<td>31–48</td>
<td>35–67</td>
</tr>
<tr>
<td>Fractures (any site)</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>History of kidney stones</td>
<td>9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73 m²)</td>
<td>76±5.3</td>
<td>81±7.1</td>
<td>84±9.3</td>
</tr>
<tr>
<td>No. of patients</td>
<td>22</td>
<td>20</td>
<td>30</td>
</tr>
</tbody>
</table>

*GFR, estimated glomerular filtration rate with MDRD formula.

Discussion

Our data point out that after an oral load of peptones, PHPT-N patients demonstrated a different calcium-regulating hormone response than PHPT patients but not different from healthy control subjects. Similarly, after an oral calcium load, PHPT-N patients showed different responses in calcium-regulating hormones than PHPT. The summary of our data evidences that PHPT-N patients respond more like healthy controls than like PHPT to oral calcitropic stimulatory tests. Lastly, to the best of our knowledge, this study is the first to explore calcitropic hormone responses to an oral peptone load in PHPT-N patients. After an oral peptone load, PTH increased more in PHPT patients than in...
healthy controls and PHPT-N patients. The latter two groups showed a similar PTH response. In PHPT patients, ionized serum calcium decreased significantly 90 min after an oral peptone load, while in PHPT-N patients and healthy controls it decreased after 120 min. However, peptones plus AH, an inhibitor of phosphate absorption, did not produce any PTH response in PHPT-N patients, while it blunted but did not completely suppress it in PHPT patients, when compared with a peptone load alone. In light of these results, it is tempting to speculate that phosphate is responsible for the increased PTH levels in PHPT patients, in whom PTH-induced modulation of CASR is partially lost (25).

**Calcium-sensing receptor**

CASR is the molecular basis for Ca\(^{2+}\) sensing by parathyroid cells, C cells (parafollicular thyroid cells), kidney cells, bone-derived cells, and intestinal cells (2, 26). PHPT is possibly caused by the loss of the inhibitory tonic action of the CASR, which normally restrains parathyroid cellular proliferation and regulates PTH gene expression (1). In chronic hypercalcemia, the calciotropic hormone response to secretagogues may be impaired through chronic inhibition of CASR activity or other mechanisms (26). Moreover, CASR normally restrains parathyroid cell proliferation and regulates PTH gene expression. Interestingly, CASR expression is reduced by about 50% in PHPT (27, 28), and medical treatment with calcimimetics, which act on the CASR, is only partially able to suppress PTH overproduction in PHPT patients (29). However, no correlation between reduced CASR expression and an increased PTH set point in PHPT has yet been demonstrated (30). Indeed, post-receptor abnormalities such as decreased levels of G-proteins and caveolin-1 may also come into play (28, 31).

**Oral calcium and PTH**

Previous works investigating oral calcium load in PHPT addressed the problem of differentiation between adenoma and parathyroid hyperplasia, showing either greater (10) or smaller (11, 12, 13) decline of serum PTH levels in PHPT patients compared with healthy controls (for a brief summary of these works, see Table 2). After the definition of PHPT-N as a clinical entity, characterized by consistently normal calcium concentrations with persistently abnormal PTH levels (5), (22) only Maruani et al. (12) demonstrated in PHPT-N patients a minimal decrease in serum PTH following an oral calcium load (25% change) despite the occurrence of hypercalcemia. However, at the present time, no comparison between PHPT-N and healthy subjects has yet been performed. For this reason, our data are the first demonstrating that after an oral calcium load PTH is suppressed in PHPT-N patients as in healthy subjects, suggesting a preserved feedback between serum calcium and PTH.

**Phosphate and PTH**

The major finding of our study regards the role of phosphate in regulating calciotropic hormone responses in PHPT and PHPT-N patients. The observation of a remarkably high response of PTH after an oral phosphate-peptone load in PHPT may be related to the observed rise in serum phosphate levels (Fig. 1). This rise may correspond to increased absorption of

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**Figure 2** Effects of an oral peptone and calcium load on phosphorus and gastrin in patients with PHPT, PHPT-N, and control subjects (CTR). *P*<0.01 vs baseline; **P**<0.01 PHPT vs PHPT-N; *P*<0.01 PHPT vs controls; **P**<0.01 PHPT-N vs controls.

**Figure 3** Effects of an oral peptone load plus AH on PTH, plasma ionized calcium, phosphorus, and gastrin in patients with PHPT, PHPT-N, and control subjects (CTR). *P*<0.01 vs baseline; *P*<0.01 PHPT vs controls; **P**<0.01 PHPT-N vs controls.
Moreover, as shown in Fig. 3, the serum phosphate level PHPT patients compared with a peptone load alone. but did not completely suppress the PTH response in plus AH, an inhibitor of phosphate absorption, blunted both PHPT and PHPT-N patients. However, peptones relation to low baseline phosphate serum levels in phosphate contained in the peptone meal (32) in aPatients with MEN1.

- **Gastrin**

Gastrin increase after an oral peptone load in PHPT patients was about half that observed in controls and PHPT-N patients. Conversely, the lack of a serum gastrin response after oral calcium load administration suggested a predominant role of amino acids in gastrin stimulation. These results confirm those previously obtained by our group (18). Furthermore, the blunted gastrin response to amino acids activating CASR observed in PHPT patients might suggest that the loss of activity of CASR could also affect other endocrine organs than parathyroid glands (e.g. gastrin-secreting cells).

**Conclusion**

This paper raises two important issues about differences in calcium-regulating hormone handling between PHPT-N and PHPT patients. The first is that the summary of our data evidences that PHP-N patients respond more like healthy controls than like PHPT to oral calciotropic stimulatory tests (both peptones and calcium). The question of whether PHPT-N patients have a true disorder, one that will cause them the same renal and skeletal complications as PHPT, remains still unsolved. Our data suggest that PHPT-N patients have a partially preserved regulatory pathway of calciotropic hormone handling compared with PHPT patients and that they may not, in fact, have autonomously secreting parathyroid glands. For this reason, they may not benefit from surgery. These oral stimulatory tests may help endocrinologists and endocrine surgeons to determine which patients with calcium levels in the normal or high/normal range indeed present autonomous secreting parathyroid glands and should be therefore considered eligible for surgery.

The second main issue regards the central role of phosphate in PTH regulation in PHPT-N patients. It has been demonstrated that in PHPT patients, PTH-induced modulation of CASR is partially lost (25); moreover, current evidence suggests the existence of a phosphate sensor in duodenum and parathyroid glands.
In light of these considerations, it is possible to hypothesize that in PHPT patients phosphate could become the main regulator of parathyroid activity, exerting its rapid effects independently of the serum calcium concentration. This could be due to an upregulation of jejunum–parathyroid axis or due to an upregulation of the ‘phosphate sensor’ on parathyroid glands or both. However, these intriguing hypotheses, as the scientific proof for the existence of the ‘phosphate sensor’, need further research. In conclusion, our results may provide useful information for a better interpretation of PHPT-N, its nature and significance, and its prognosis and treatment. We suggest that the oral peptone test could be added to the diagnostic evaluation of PHPT-N to prevent patients from receiving as a first-step expensive and invasive diagnostic tests. In case of absent response to peptones, these patients should have their serum calcium levels assessed twice a year so that further development of PHPT can be treated promptly, in accordance with recent guidelines (5, 8, 9, 38).

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

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