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## Intestinal $\text{Ca}^{2+}$ absorption revisited: A molecular and clinical approach

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### Abstract

$\text{Ca}^{2+}$  has an important role in the maintenance of the skeleton and is involved in the main physiological processes. Its homeostasis is controlled by the intestine, kidney, bone and parathyroid glands. The intestinal  $\text{Ca}^{2+}$  absorption occurs mainly *via* the paracellular and the transcellular pathways. The proteins involved in both ways are regulated by calcitriol and other hormones as well as dietary factors. Fibroblast growth factor 23 (FGF-23) is a strong antagonist of vitamin D action. Part of the intestinal  $\text{Ca}^{2+}$  movement seems to be vitamin D independent. Intestinal  $\text{Ca}^{2+}$  absorption changes according to different physiological conditions. It is promoted under high  $\text{Ca}^{2+}$  demands such as growth, pregnancy, lactation, dietary  $\text{Ca}^{2+}$  deficiency and high physical activity. In contrast, the intestinal  $\text{Ca}^{2+}$  transport decreases with aging. Oxidative stress inhibits the intestinal  $\text{Ca}^{2+}$  absorption whereas the antioxidants counteract the effects of prooxidants leading to the normalization of this physiological process. Several pathologies such as celiac disease, inflammatory bowel diseases, Turner syndrome and others occur with inhibition of intestinal  $\text{Ca}^{2+}$  absorption, some hypercalciurias show  $\text{Ca}^{2+}$  hyperabsorption, most of these alterations are related to the vitamin D endocrine system. Further research work should be accomplished in order not only to know more molecular details but also to detect possible therapeutic targets to ameliorate or avoid the consequences of altered intestinal  $\text{Ca}^{2+}$  absorption.

**Key words:**  $\text{Ca}^{2+}$  absorption; Transcellular pathway; Paracellular pathway; Hormones; Dietary calcium; Physiological conditions; Pathological alterations

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**Core tip:** The intestinal  $\text{Ca}^{2+}$  absorption occurs mainly *via* the paracellular and the transcellular pathways. Both ways are regulated by calcitriol and other hormones as well as dietary factors. Fibroblast growth factor 23 (FGF-23) is a strong antagonist of vitamin



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D action. Part of the intestinal Ca<sup>2+</sup> movement seems to be vitamin D independent. Intestinal Ca<sup>2+</sup> absorption changes according to different physiological conditions. Oxidative stress inhibits the intestinal Ca<sup>2+</sup> absorption whereas the antioxidants counteract the prooxidant effects. Most diseases that occur with altered intestinal Ca<sup>2+</sup> absorption is related to changes in the vitamin D endocrine system. Further research could clarify many unknown points in this subject.

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## INTRODUCTION

Ca<sup>2+</sup> plays a relevant role in the skeleton, being the bones the storage site of 99% of total body Ca<sup>2+</sup>, mainly in the form of hydroxyapatite crystals<sup>[1]</sup>. Ca<sup>2+</sup> is essential for the acquisition of an optimal peak bone mass within the first two decades, as well as for the maintenance of bone mineral density (BMD) in adulthood<sup>[2]</sup>. Apart from skeletal mineralization, this divalent cation is an important intracellular messenger and it actively participates in multiple physiological functions such as nerve excitation and transmission, muscle and cardiac contraction, blood coagulation, gland secretion and enzyme activation, among others. It is also implicated in cell differentiation and apoptosis as well as in immune response, thus having a significant role in tumorigenesis and cancer development<sup>[3]</sup>. This vast scope of functions raises the need for a homeostatic regulatory system. Extracellular and metabolic Ca<sup>2+</sup> homeostasis is achieved by the concerted action of several Ca<sup>2+</sup> regulating hormones: Parathyroid hormone (PTH), 1,25(OH)<sub>2</sub>D<sub>3</sub> or calcitriol and calcitonin (CT). Estrogen, insulin-like growth factor (IGF-1), prolactin (PRL) and fibroblast growth factor (FGF-23) also participate in Ca<sup>2+</sup> regulation<sup>[4]</sup>.

Intestinal absorption is the unique way for Ca<sup>2+</sup> to enter the organism from dietary nutrients. It occurs *via* two different mechanisms. When luminal Ca<sup>2+</sup> is higher than that in the plasma, Ca<sup>2+</sup> predominantly enters the intestine *via* the paracellular pathway through tight junctions between neighbouring enterocytes<sup>[5]</sup>. This is a passive non saturable transport, which takes place all along the small intestine without significant variations. In contrast, when luminal Ca<sup>2+</sup> is lower than plasmatic Ca<sup>2+</sup>, the cation is actively absorbed *via* the transcellular pathway, which is an active saturable system that prevails in the duodenum<sup>[5,6]</sup>. Both mechanisms are regulated by different endocrine and non-endocrine factors.

Intestinal Ca<sup>2+</sup> absorption in humans reaches approximately 35% of dietary load<sup>[7]</sup>. The amount of Ca<sup>2+</sup> absorbed mainly depends on the quantity of Ca<sup>2+</sup> consumed, the transit time in different parts of the gut and the solubility of Ca<sup>2+</sup>, which is inversely related to luminal pH<sup>[8]</sup>. Even though the absorption is more efficient in the duodenum and jejunum, where pH is lower, the amount of Ca<sup>2+</sup> absorbed is greater in the ileum, since intestinal content remains ten times longer in this portion, which is finally responsible for almost 65% of Ca<sup>2+</sup> absorption<sup>[9]</sup>.

## MOLECULAR MECHANISMS OF INTESTINAL Ca<sup>2+</sup> ABSORPTION

### Paracellular pathway

This absorptive route comprises the entrance of Ca<sup>2+</sup> through electrically-charged watery space delimited by two neighbouring enterocytes. As it is the result of passive diffusion, this movement of the cation depends on Ca<sup>2+</sup> concentration and voltage across intestinal epithelium. Rats fed a Ca<sup>2+</sup>-replete diet and humans show a chemical gradient which is favorable to the inward movement of the cation, since free Ca<sup>2+</sup> is present in the duodenal lumen in a concentration of 2-6 mmol/L, compared to the much lower concentration in the interstitial fluid or plasma (1.25 mmol/L)<sup>[9,10]</sup>. As to electrical potential, the plasma is positive compared to the lumen, which would determine a secretion movement and exit of Ca<sup>2+</sup>. However, since this voltage

gradient is very low, (2.5 mV), there is no outward flow of the cation due to electrodiffusion<sup>[11]</sup>. Apart from chemical gradient,  $\text{Ca}^{2+}$  together with other ions and small hydrophilic molecules enter through paracellular space along with the stream of water. This solvent drag-induced mechanism depends on the activation of sodium-glucose co-transporter 1 (SGLT-1) and  $\text{Na}^+/\text{K}^+$ -ATPase. Sodium, which enters through SGLT-1 and other sodium-coupled apical transporters, is pumped into the paracellular space by  $\text{Na}^+/\text{K}^+$ -ATPase, thus increasing sodium paracellular concentration and consequently augmenting osmotic water flow through this space. For this reason luminal glucose and galactose, which are substrates of SGLT-1, increase duodenal  $\text{Ca}^{2+}$  absorption<sup>[12]</sup>. Finally, duodenal epithelium has a preferential selectivity for small mono or divalent cations, such as  $\text{Na}^+$  or  $\text{Ca}^{2+}$ , over larger or negative ions. This selectivity depends on some proteins such as occludin and claudins (CLDNs) in the tight junctions, which are specialized membrane domains in the apical region of enterocytes. CLDNs 2, 12, and 15 are associated with intestinal  $\text{Ca}^{2+}$  entering<sup>[13,14]</sup>. CLDNs 1 and 5 have sealing functions that could also diminish  $\text{Ca}^{2+}$  transport<sup>[15,16]</sup>. The expression of four candidate genes of the paracellular pathway, CLDN2 and tight junction proteins 1, 2, and 3, has been shown to be enhanced in the small intestine of laying hens after sexual maturity<sup>[6]</sup>.

### **Transcellular pathway**

This active transport is carried out in three main steps; firstly,  $\text{Ca}^{2+}$  enters the enterocytes passively across the apical membranes. Afterwards, it binds to intracytoplasmatic proteins, which transfer the cation to the basolateral membrane (BLM). Finally, plasma membrane  $\text{Ca}^{2+}$ -ATPase ( $\text{PMCA}_{1b}$ ) is the main  $\text{Ca}^{2+}$  transporter that extrudes the cation by primary active transport, at the expense of stored ATP.

### **Entrance of $\text{Ca}^{2+}$ across the brush border membrane**

Brush border membrane (BBM) is the first barrier that  $\text{Ca}^{2+}$  has to overcome in its way from intestinal lumen into the blood. Since  $\text{Ca}^{2+}$  is not able to move freely across the lipid bilayer of the plasma membrane, intestinal absorptive cells express some  $\text{Ca}^{2+}$  transporters. There are two epithelial  $\text{Ca}^{2+}$  channels that favor the passive transport of the cation across the apical membrane into enterocyte cytoplasm: Transient receptor potential vanilloid 5 (TRPV5) also known as  $\text{ECaC1}$  or  $\text{CaT2}$ , and transient receptor potential vanilloid 6 (TRPV6); also known as  $\text{ECaC2}$  or  $\text{CaT1}$ <sup>[2]</sup>. TRPVs are very important for maintaining blood  $\text{Ca}^{2+}$  levels in higher organisms, humans included. They are localized in apical membrane of  $\text{Ca}^{2+}$ -transporting epithelial tissues and respond to  $1,25(\text{OH})_2\text{D}_3$ . They are structurally very similar since they share 75% of aminoacid identity. However, they differ in their distribution: TRPV5 expression is almost restricted to the kidney<sup>[17]</sup> and determines the level of urinary excretion of  $\text{Ca}^{2+}$  whereas TRPV6 shows intense expression in the intestine, thus being particularly important in intestinal  $\text{Ca}^{2+}$  absorption. More ubiquitously expressed, TRPV6 is also present in the kidney, placenta, epididymis and exocrine glands among other tissues<sup>[17,18]</sup>. Apical entrance of  $\text{Ca}^{2+}$  through TRPV6 is warranted by the favourable inwardly oriented electrochemical gradient for  $\text{Ca}^{2+}$ , usually in low intracellular concentrations (100 nmol/L)<sup>[19]</sup>.

Although TRPV6 is central for active  $\text{Ca}^{2+}$  absorption, studies in TRPV6 KO-mice have demonstrated that some  $\text{Ca}^{2+}$  is still absorbed when TRPV6 is absent<sup>[20]</sup>. Even more, the stimulating effect of  $1,25(\text{OH})_2\text{D}_3$  on intestinal  $\text{Ca}^{2+}$  absorption is partially preserved in these animals<sup>[21]</sup>. In the same line, Woudenberg-Vrenken *et al*<sup>[22]</sup> obtained similar results when evaluating transepithelial  $\text{Ca}^{2+}$  absorption in a mouse-model carrying a nonfunctional TRPV6. They found that mice experimentally modified in one critic aminoacid residing in the pore site of the channel had lower intestinal  $\text{Ca}^{2+}$  uptake than wild-type controls and interestingly, showed a compensatory upregulation of TRPV5; however, insufficient to completely correct the diminished  $\text{Ca}^{2+}$  absorption. These findings strongly suggest that some other molecules may participate in the apical crossing of the electrolyte.

Another type of  $\text{Ca}^{2+}$  channel is present in the surface of enterocytes,  $\text{Ca}_v1.3$ , an apical L-type voltage-dependent channel present from the duodenum to the ileum, which might have a complementary role in  $\text{Ca}^{2+}$  permeation through TRPV6. The latter channel predominates in the duodenum, is activated by hyperpolarization, mainly overnight and between meals, and depends on vitamin D. In contrast,  $\text{Ca}_v1.3$ , predominates in mid-ileum, is activated when membrane is depolarized, mainly during postprandial active digestion, and is not modulated by vitamin D<sup>[23]</sup>.

### **Intracellular $\text{Ca}^{2+}$ crossing from apical to BLM**

Once  $\text{Ca}^{2+}$  has passively entered the enterocyte, it binds to calbindins (CBs) and is subsequently transferred to BLM. All these proteins have a high  $\alpha$ -helical content and share EF-hand structures with a helix-loop-helix sites that constitute the  $\text{Ca}^{2+}$ -binding

domains. These EF-hand motifs are held together in a single globular fold *via* hydrophobic interactions<sup>[24]</sup>. CB<sub>9k</sub> is present in the intestine of mammals, being highly abundant in the duodenum and gradually decreasing downwardly to become undetectable in distal ileum and colon<sup>[25]</sup>. CBs belong to a superfamily of Ca<sup>2+</sup>-binding proteins that also includes calmodulin and troponin C<sup>[26]</sup>. It has been shown that CB<sub>28k</sub> undergoes structural changes upon Ca<sup>2+</sup> binding, which is indicative of a Ca<sup>2+</sup>-sensing protein<sup>[27]</sup>.

Even though Ca<sup>2+</sup> can cross the aqueous cytoplasmic environment at a higher rate than when associated with proteins<sup>[28]</sup>, its binding to CBs prevents the free flow of Ca<sup>2+</sup>, helping to maintain intracellular Ca<sup>2+</sup> lower than 10<sup>-7</sup> mol/L. This Ca<sup>2+</sup> buffering is extremely important since it prevents the potential deleterious pro-apoptotic effect of the cation, which has been confirmed in different tissues<sup>[29]</sup>.

Since excessive free-ionized Ca<sup>2+</sup> next to the BBM could deactivate TRPV6 restricting Ca<sup>2+</sup> entry, the Ca<sup>2+</sup> transferring function of these proteins may also contribute to warranting a persistent Ca<sup>2+</sup> entry through the apical membrane<sup>[30]</sup>. In this direction, Song *et al*<sup>[31]</sup> have found that both TRPV6 and CB<sub>9k</sub> are similarly regulated, both being induced at weaning or under low Ca<sup>2+</sup> diets.

The expression of CB<sub>9k</sub> in duodenal enterocytes strongly depends on 1,25(OH)<sub>2</sub>D<sub>3</sub><sup>[32]</sup>, as also does the expression of other Ca<sup>2+</sup>-binding proteins such as CB<sub>28k</sub>, calmodulin, parvalbumin and sorcin<sup>[33]</sup>, although the significance of their contribution to Ca<sup>2+</sup> transferring remains to be specified. However, similarly to what we have referred for TRPV6, CB<sub>9k</sub>-KO mice partially preserve Ca<sup>2+</sup> translocation capacity<sup>[20]</sup>, which reinforces the potential role in cytoplasmatic Ca<sup>2+</sup> translocation of several proteins other than CB<sub>9k</sub>. In this sense, Teerapornpuntakit *et al*<sup>[34]</sup> have found an upregulation of CB<sub>28k</sub> and parvalbumin expression in pregnancy and lactation, as a complementary mechanism to supply for the high demand of Ca<sup>2+</sup> in these physiological conditions. Hwang *et al*<sup>[35]</sup> have observed an increase in the expression of most tight junction genes in the duodenum of normally fed CB<sub>9k</sub>-KO mice compared to wild-type controls. These findings suggest that the transcellular Ca<sup>2+</sup>-binding proteins may also exert some regulatory effect on paracellular Ca<sup>2+</sup> absorption, suggesting that active and passive Ca<sup>2+</sup> transport pathways may function cooperatively.

### Ca<sup>2+</sup> exit across the BLM

The active Ca<sup>2+</sup> transport is required to overcome the unfavourable electrochemical gradient for Ca<sup>2+</sup> across the BLM. PMCA<sub>1b</sub> and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX1) are the two proteins in charge of extruding Ca<sup>2+</sup> out of the enterocyte, thus completing the transcellular Ca<sup>2+</sup> absorption.

PMCA<sub>1b</sub>, responsible for almost 80% of Ca<sup>2+</sup> extrusion<sup>[36]</sup>, is highly active in the duodenum, as detected in rats and it is a primary active transporter able to hydrolyze directly ATP to transport the cation<sup>[37]</sup>. The expression and activity of this pump is higher in enterocytes from the tip of the villi than in those from the crypt. This difference goes in line with the concept that mature enterocytes show a greater efficiency in transcellular Ca<sup>2+</sup> movement<sup>[38]</sup>. NCX1, in contrast, is a secondary active transporter, coupled with Na<sup>+</sup>/Ca<sup>2+</sup>-ATPase, which creates a sodium gradient for NCX1-mediated Ca<sup>2+</sup> efflux. It has a Na<sup>+</sup>/Ca<sup>2+</sup> stoichiometry of 3:1 and it can either extrude or intrude Ca<sup>2+</sup> depending on cation gradients and the potential across the plasma membrane<sup>[39]</sup>. It appears to be responsible for approximately 20% of basolateral uphill Ca<sup>2+</sup> extrusion from the enterocyte into blood stream<sup>[37]</sup>. Despite the importance of NCX1 in several tissues such as cardiac muscle, vascular smooth muscle and the nerves<sup>[40-42]</sup>, the lower implication of this transporter in Ca<sup>2+</sup> absorption as compared to PMCA<sub>1b</sub> activity caused NCX1 to be neglected in many studies concerning the intestine.

Basolateral Ca<sup>2+</sup> absorption is closely related to the uptake of the cation in the apical membrane and its intracellular translocation. In this sense, a linear relationship between apical uptake and PMCA activity has been found in the duodenum of male rats<sup>[43]</sup>. In addition, CB<sub>9k</sub> and CB<sub>28k</sub>, calmodulin and parvalbumin activate PMCA<sup>[44,45]</sup>. This concerted mechanism contributes to avoiding intracellular accumulation of Ca<sup>2+</sup>, which would block Ca<sup>2+</sup> entry from the lumen and could lead to apoptosis.

## REGULATION OF INTESTINAL CALCIUM ABSORPTION

### Hormonal regulation of calcium absorption

**Vitamin D:** 1,25(OH)<sub>2</sub>D<sub>3</sub>, the active metabolite of vitamin D, is the main regulating hormone of intestinal Ca<sup>2+</sup> absorption. It induces structural and functional modifications in enterocytes and helps to enhance both transcellular and paracellular pathways, either by genomic or nongenomic actions<sup>[46,47]</sup>. 1,25(OH)<sub>2</sub>D<sub>3</sub> can reach the



intestinal target coming from two different sources: Either from the plasma, once its synthesis has been completed by 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylase (CYP27B1) in renal proximal tube (endocrine source)<sup>[48]</sup> or from *de novo* synthesis in the cytoplasm of the enterocyte, performed by a duodenal 1 $\alpha$ -hydroxylase (intracrine source)<sup>[49]</sup>.

This calcitropic lipophilic vitamin passes through the plasma membrane and binds to vitamin D receptor (VDR), its nuclear receptor. Once bound to the ligand, VDR forms a heterodimer with retinoid X receptor (RXR) and the new 1,25(OH)<sub>2</sub>D<sub>3</sub>-VDR- RXR complex functions as a transcription factor which binds to different vitamin D response elements in various target genes<sup>[50]</sup>. This determines a significant increase in the expression of all Ca<sup>2+</sup> transporting proteins in the enterocyte: TRPV 6, CB<sub>9K</sub>, PMCA<sub>1b</sub> and NCX1, as has been demonstrated in animal models and humans<sup>[2,4,49,51-54]</sup>. Vitamin D-mediated Ca<sup>2+</sup> absorption has mainly been studied in the proximal intestine, where Ca<sup>2+</sup> is more efficiently absorbed. However, Christakos *et al*<sup>[30]</sup> have recently studied this process in mice with transgenic expression of VDR exclusively in the ileum, cecum and colon of VDR KO mice. Interestingly, these animals did not present the abnormalities in Ca<sup>2+</sup> homeostasis and bone mineralization usually seen in VDR KO mice. These findings emphasize the importance of 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated Ca<sup>2+</sup> absorption in the distal intestine<sup>[55]</sup>.

Apart from these Ca<sup>2+</sup>-transporting proteins, 1,25(OH)<sub>2</sub>D<sub>3</sub> can regulate other important genes in Ca<sup>2+</sup> metabolism, such as the one of 24-hydroxylase (CYP24A1) which converts 1,25(OH)<sub>2</sub>D<sub>3</sub> into 1,24,25(OH)<sub>3</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> into 24,25(OH)<sub>2</sub>D<sub>3</sub>, and CYP27B1, involved in the renal synthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub><sup>[56]</sup>, but also expressed in the intestine<sup>[57]</sup> and parathyroid gland<sup>[58]</sup>.

In addition to the genomic action described, there is some evidence that 1,25(OH)<sub>2</sub>D<sub>3</sub> also binds to a plasma membrane receptor (MARRS: Membrane-associated, rapid response steroid-binding protein), which, in turn, activates other second messenger systems such as phospholipase A2 and protein kinase C<sup>[59-61]</sup>. Details of the underlying molecular mechanism of 1,25(OH)<sub>2</sub>D<sub>3</sub>-MARRS and its rapid minute-to-minute regulatory capacity remain to be elucidated.

Even though the transcellular pathway has been the focus of most studies concerning the effect of calcitriol on intestinal Ca<sup>2+</sup> absorption, vitamin D has proved to exert a positive effect on the paracellular absorptive route as well. 1,25(OH)<sub>2</sub>D<sub>3</sub> is able to change the permeability and selectivity of the tight junctions by altering certain crucial proteins such as CLDNs 2 and 12. This would help to enhance passive diffusion of Ca<sup>2+</sup><sup>[62]</sup>.

Rexhepaj *et al*<sup>[63]</sup> have observed that 1,25(OH)<sub>2</sub>D<sub>3</sub> could also stimulate Na<sup>+</sup>/Ca<sup>2+</sup>-ATPase and SGLT, and consequently increase water-movement through the junction, thus carrying more Ca<sup>2+</sup> inwardly with the flow. Tudpor *et al*<sup>[64]</sup> have demonstrated a dose dependent increase in solvent drag-induced Ca<sup>2+</sup> movement one hour after direct exposure of rats to 10-100 nmol/L 1,25(OH)<sub>2</sub>D<sub>3</sub>. This rapid effect, abolished by inhibitors of phosphatidylinositol 3-kinase, protein kinase C, and MEK, would be mediated by nongenomic mechanisms involving 1,25(OH)<sub>2</sub>D<sub>3</sub>-MARRS.

It has also been reported that 1,25(OH)<sub>2</sub>D<sub>3</sub> downregulates intestinal cadherin-17 (involved in cell-to-cell contact) and aquaporin-8 (associated with epithelial selectivity towards cations), which might also affect the Ca<sup>2+</sup> absorption<sup>[20,65]</sup>.

**PTH:** PTH, a hypercalcemic hormone secreted by parathyroid glands, is the other classical hormone known to exert a positive regulatory effect on intestinal Ca<sup>2+</sup> absorption. However, this stimulatory effect is achieved indirectly after increasing CYP27B1 transcription for 1 $\alpha$ -hydroxylase, the renal enzyme that completes the synthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the kidney. As a result, 1,25(OH)<sub>2</sub>D<sub>3</sub> production augments. In addition, PTH also suppresses the transcription of CYP24A1 that codifies for 24-hydroxylase, a renal enzyme which degrades 1,25(OH)<sub>2</sub>D<sub>3</sub> by converting it into 1,24,25(OH)<sub>3</sub>D<sub>3</sub><sup>[66]</sup>. Both actions lead to an increase in plasmatic 1,25(OH)<sub>2</sub>D<sub>3</sub>, which in turn enhances Ca<sup>2+</sup> absorption as we have already revised.

**Thyroid hormones:** Thyroxine (T4) and triiodothyronine (T3) are known to regulate metabolism in general. Overproduction of T4 or T3 in the context of hyperthyroidism can lead to hypercalcemia due to an excessive bone turnover and consequently lead to bone demineralization<sup>[67,68]</sup>. However, there is some evidence that thyroid hormones would cooperate with vitamin D by increasing the genomic actions of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the intestine<sup>[69]</sup>. Cross *et al*<sup>[70]</sup> have shown that calcitriol added to cultures of 20-day-old embryonic chick small intestine, stimulated Na<sup>+</sup> uptake. The calcitriol-mediated increase in Na<sup>+</sup> uptake appeared to be related to increased tight-junctional or paracellular permeability<sup>[70]</sup>. It can be speculated that this effect could favor the paracellular entry of Ca<sup>2+</sup> as well. More recently, Kumar *et al*<sup>[71]</sup> have shown that Ca<sup>2+</sup> influx in BBM vesicles was higher in enterocytes from hyperthyroid rats as compared to those of hypothyroid ones. The authors have proposed that this could be related to

a change in membrane fluidity induced by thyroid hormones. Similarly, they have also observed that efflux of calcium across BLM was also higher in hyperthyroid rats. This difference was associated with a higher NCX1 activity triggered by thyroid hormones, possibly through the cAMP-mediated pathway. cAMP is a potent activator of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger and it was significantly higher in intestinal mucosa of hyperthyroid rats as compared to euthyroid animals. In addition to these actions, thyroid treatment increases serum PTH and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels, which contributes to enhancing Ca<sup>2+</sup> absorption indirectly through vitamin D<sup>[7]</sup>.

**Growth hormone:** Growth hormone (GH) has a central role in longitudinal bone growth and mineralization during childhood and adolescence. However, this metabolic hormone has receptors in most tissues and exerts various actions apart from skeletal growth. There is evidence that GH has proliferative effects on intestinal mucosa<sup>[72]</sup>. GH has been used to treat inflammatory bowel disease in pediatric<sup>[73]</sup> and adult patients<sup>[74]</sup>. Interestingly, FDA has approved the use of recombinant human GH to treat short bowel syndrome, where it improves absorption of carbohydrates, amino acids and fats<sup>[75,76]</sup>. GH can also stimulate intestinal Ca<sup>2+</sup> absorption, which would occur indirectly by activating renal CYP27B1 and consequently increasing serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels<sup>[77]</sup>. It has been demonstrated that GH can prevent the loss of intestinal VDR in ovariectomized (OVX) rats<sup>[78]</sup>, which would suggest that it could increase intestinal sensitivity to 1,25(OH)<sub>2</sub>D<sub>3</sub> by regulating tissue VDR levels<sup>[79]</sup>. However, the positive effect of GH on Ca<sup>2+</sup> absorption would not be exclusively dependent on vitamin D. Fleet *et al*<sup>[80]</sup> have shown that GH increases intestinal Ca<sup>2+</sup> absorption and duodenal CB9k levels in aged rats without increasing serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels. Analogous results have been found in humans. In adult men, Ca<sup>2+</sup> absorption has a positively correlation with IGF-1 and age-related decline in IGF-1 has a negative impact on Ca<sup>2+</sup> absorption that could not be justified by a decrease in serum 1,25(OH)<sub>2</sub>D<sub>3</sub><sup>[81]</sup>.

**Estrogens:** There is evidence that post-menopausal women experiment an increase in bone resorption together with a reduction in Ca<sup>2+</sup> absorption and an increase in urinary Ca<sup>2+</sup> excretion as a consequence of estrogen loss<sup>[82,83]</sup>. Post-menopausal low estrogen levels have been associated with reduced serum 1,25(OH)<sub>2</sub>D<sub>3</sub><sup>[84]</sup>. However, OVX rats have no reduction in serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels<sup>[85]</sup>, which would suggest the implication of vitamin D independent mechanisms. Thus, O'Loughlin *et al*<sup>[86]</sup> observed that estradiol replacement in OVX rats increases intestinal Ca<sup>2+</sup> absorption without stimulation of circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels. In the same line, van Abel *et al*<sup>[87]</sup> found increased duodenal gene expression of TRPV5, TRPV6, CB<sub>9k</sub>, and PMCA1b in OVX rats treated with estradiol. In order to determine whether this stimulatory effect on Ca<sup>2+</sup> transporting proteins was calcitriol-dependent, they used CYP27B1 KO mice and found that estradiol treatment increased mRNA levels of duodenal TRPV6. In contrast, Gennari *et al*<sup>[88]</sup> found that oophorectomy in young women reduces the intestinal Ca<sup>2+</sup> absorption induced by vitamin D, which was reversed by estrogen repletion. Other studies suggest the possibility of a deficient intestinal responsiveness to vitamin D due to a reduction of VDR levels<sup>[78,89,90]</sup>. However, the loss of intestinal VDR levels following estrogen reduction could not be confirmed in all studies<sup>[91]</sup>.

Cell-culture experiments suggest that estrogen is able to reverse the decline in the efficiency of intestinal Ca<sup>2+</sup> absorption at menopause onset<sup>[92]</sup>, but the mechanisms that underlie this effect remain to be elucidated. Estrogen receptor alpha (ERα) KO mice showed a decrease in duodenal TRPV6 mRNA expression, without modification in CB<sub>9k</sub>, PMCA1b, and VDR levels. Therefore, it seems that the genomic effects of estrogen on mice intestinal mucosa are mainly mediated by ERα<sup>[93]</sup>. However, Nie *et al*<sup>[94]</sup> have recently reported that estrogen regulates duodenal Ca<sup>2+</sup> absorption through differential effects of ERα and ERβ on TRPV6 and PMCA1b expressions in duodenal epithelial cells, respectively.

**PRL:** The main lactogenic hormone, PRL, is elevated during pregnancy and lactation. Apparently, this pituitary hormone is able to enhance Ca<sup>2+</sup> absorption in order to supply calcium for milk production. It has been shown that PRL enhances CYP27B1 protein expression and increases levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> during lactation, a moment when there is an increased Ca<sup>2+</sup> requirement for the neonate<sup>[95]</sup>. However, its calcitropic action is not only achieved *via* vitamin D. It has been shown that PRL stimulates active intestinal Ca<sup>2+</sup> transport in vitamin D-deficient rats<sup>[96]</sup>. Charoenphandhu *et al*<sup>[11]</sup> demonstrated that PRL directly stimulates active duodenal Ca<sup>2+</sup> transport. Wongdee *et al*<sup>[97]</sup> observed that lactating rats exhibit some adaptive changes in their intestinal mucosa tending to increase the absorptive surface area. These rats have larger duodenal, jejunal and ileal villous as well as deeper cecal crypts than age-matched nulliparous rats. These histological modifications were diminished

by bromocriptine, an inhibitor of pituitary PRL release. PRL also upregulated TRPV6 and PMCA<sub>1b</sub> in the duodenum of lactating rats. These changes were associated with a compensatory increase in FGF-23 expression, a local negative regulator of  $\text{Ca}^{2+}$  absorption, presumably to prevent  $\text{Ca}^{2+}$  hyperabsorption. Bromocriptine also manages to abolish FGF-23 increment, confirming it was induced by PRL. In addition, it has been suggested that PRL has also a stimulating effect on paracellular pathway by upregulating CLDN 15 in the tight junctions<sup>[98]</sup>.

**FGF-23:** It is a glycoprotein secreted by osteocytes and osteoclasts and regulated by plasma levels of  $1,25(\text{OH})_2\text{D}_3$  and Pi. The enhancement of these regulators leads to the serum increase in FGF-23, which in turn reduces the concentration of  $1,25(\text{OH})_2\text{D}_3$  by inhibiting  $1\alpha$ -hydroxylase and stimulating  $24\alpha$ -hydroxylase. As for Pi, FGF-23 increases its renal excretion<sup>[99]</sup>.

FGF-23 has been indicated as a vitamin D antagonist in intestinal absorption of  $\text{Ca}^{2+}$ . Khuituan *et al*<sup>[32]</sup> have demonstrated that intravenous administration of FGF-23 to male rats abolished the increase in intestinal absorption of  $\text{Ca}^{2+}$  caused by the injection of  $1,25(\text{OH})_2\text{D}_3$ . However, the inhibitory effect of FGF-23 could not be observed in the absence of the previous supply of  $1,25(\text{OH})_2\text{D}_3$ . The mechanisms underlying the effect of FGF-23 would be related to the decrease in the gene expression of TRPV5, TRPV6 and CB<sub>9k</sub> caused by this phosphaturic hormone. In this same work, the presence of FGFR1-4 in the BLM of rat enterocytes was confirmed. However, their functions are unclear since the direct exposure of the intestinal epithelium to FGF-23 did not cause alterations.

FGF-23 also blocks the stimulatory effect of  $1,25(\text{OH})_2\text{D}_3$  on the paracellular pathway of intestinal  $\text{Ca}^{2+}$  absorption<sup>[100]</sup>. Since vitamin D favors this process by increasing water flow across paracellular space and consequently dragging solutes as  $\text{Ca}^{2+}$ , it has been proposed that FGF-23 could decrease the water flow and the dragging of this cation.

The activation of the mechanisms mediated by FGF-23 would be crucial to avoid the  $\text{Ca}^{2+}$  hyperabsorption. Therefore, it was thinkable that a molecule that senses extracellular  $\text{Ca}^{2+}$  as the calcium sensing receptor (CaSR) would play an important role. In fact, Rodrat *et al*<sup>[101]</sup> have demonstrated that CaSR was involved in the inhibition of intestinal  $\text{Ca}^{2+}$  absorption mediated by FGF-23. According to their findings in a cell monolayer, the use of allosteric inhibitors of CaSR could reverse the inhibitory effect of FGF-23 on the stimulation of  $\text{Ca}^{2+}$  transport triggered by  $1,25(\text{OH})_2\text{D}_3$ .

**Glucocorticoids:** The negative side effects of glucocorticoid (GC) treatment on bone health are well known. Impaired function and number of osteoblasts and osteoclasts, high resorption rate, deficiency in mineralization are some of the effects of chronic treatment that lead to the development of GC-induced osteoporosis (GIO)<sup>[102]</sup>. GIO is also partially due to the alterations that GC produces in intestinal  $\text{Ca}^{2+}$  absorption. Van Cromphaut *et al*<sup>[103]</sup> evaluated the effect of dexamethasone treatment on the gene expression of proteins involved in the intestinal absorption of the cation. They did not find alterations in gene expression or  $\text{Ca}^{2+}$  absorption in the treated mice, justifying the absence of effects with the short treatment duration. Kim *et al*<sup>[104]</sup> determined that a single dose of dexamethasone increased the gene expression of TRPV6 and CB<sub>9k</sub>, while when it was given for 5 days, it led to a reduction in the expression of both genes. In concordance with these results, mRNA levels for duodenal VDR increased on day one, while they were reduced after 5 days of treatment. Zhang *et al*<sup>[105]</sup> observed reduced protein expression of TRPV6 and CB<sub>9k</sub> in the intestine of male mice injected with dexamethasone 3 times a week, for 12 weeks, effect that was accompanied by hypercalciuria and reduction in serum  $\text{Ca}^{2+}$  levels. Although the role of GC in intestinal  $\text{Ca}^{2+}$  absorption is not clear, the results presented would allow infer a certain negative effect of GC on cation transfer from the lumen to the interstitium.

**CT:** The role of CT on intestinal  $\text{Ca}^{2+}$  absorption is controversial. Some studies have suggested that CT inhibits the process; in contrast, others indicate that has a stimulatory effect. Swaminathan *et al*<sup>[106]</sup> have demonstrated that CT may produce an inhibitory effect at low doses, whereas high doses increase the intestinal  $\text{Ca}^{2+}$  absorption. CT effect could be mediated by the vitamin D endocrine system, since it has been demonstrated in diabetic rats that CT increases  $1,25(\text{OH})_2\text{D}_3$  synthesis at renal level<sup>[107]</sup>. Use of CT has been suggested to treat patients with  $\beta$ -thalassemia because they usually have low plasma levels of this hormone. CT chronic use has benefited osteoporosis associated with thalassemia, not only for its inhibitory effect on osteoclasts but also for the possible role in the  $1,25(\text{OH})_2\text{D}_3$  synthesis<sup>[108]</sup>.

### Regulation of intestinal $\text{Ca}^{2+}$ absorption by dietary calcium

The main dietary factor that can modify intestinal  $\text{Ca}^{2+}$  absorption is calcium itself.

Low-calcium uptake could eventually produce hypocalcemia, which would augment PTH secretion leading to stimulate vitamin D endocrine system and demineralize bone<sup>[109]</sup>. On the other hand, high calcium diets and calcium hyperabsorption could increase cardiovascular risk associated with vascular calcification, nephrolithiasis and dementia, among other conditions<sup>[110,111]</sup>. Since the gut is the only gate for Ca<sup>2+</sup> uptake, it is subjected to both local and systemic regulations, which protect against either insufficient or excessive Ca<sup>2+</sup> absorption<sup>[112]</sup>. Low calcium diets enhance serum levels of vitamin D and, consequently, activate the endocrine actions of this vitamin. Thus, a chronic dietary Ca<sup>2+</sup> deficiency increases all transcellular pathway genes and proteins<sup>[109,113,114]</sup>, and increases the activity of the intestinal PMCA<sub>1b</sub> and NCX1 all along the villus, independently of cell maturation degree<sup>[38]</sup>. Benn *et al*<sup>[20]</sup> have gone further to demonstrate that this adaptive increase in Ca<sup>2+</sup> absorption is present even in TRPV6 KO and CB<sub>9k</sub> KO mice, suggesting that TRPV6, which has been postulated as the rate-limiting factor in transcellular pathway, may not be so or it may be successfully replaced by other factors able to partially compensate its function. In our laboratory, we have observed in animals under low Ca<sup>2+</sup> diets that the increment in Ca<sup>2+</sup> transport is accompanied by a concomitant increase in the activity of intestinal alkaline phosphatase (IAP), a marker enzyme of enterocytic differentiation that may have a role in intestinal Ca<sup>2+</sup> absorption<sup>[38]</sup>. Brun *et al*<sup>[115]</sup> have reported that luminal Ca<sup>2+</sup> concentration increases the activity of IAP and simultaneously decreases the percentage of Ca<sup>2+</sup> absorption, functioning as a minute-to-minute local regulatory mechanism of Ca<sup>2+</sup> entry, independent of vitamin D. This would limit an excessive Ca<sup>2+</sup> intake secondary to dietary calcium restriction, thus preventing possible acute toxic effects. This regulatory mechanism may probably be one of the reasons why high Ca<sup>2+</sup> intake (1500 mg/d) was not followed by a significant increase in Ca<sup>2+</sup> absorption in a clinical trial<sup>[116,117]</sup>, as it would have been expected from the positive effect of stimulated vitamin D endocrine system. Interestingly, L-Phenylalanine, an inhibitor of IAP, prevented this regulatory effect and Ca<sup>2+</sup> uptake remained increased. A more recent study showed that IAP activity induced by luminal calcium concentration provoked changes in luminal pH that could modulate intestinal Ca<sup>2+</sup> absorption<sup>[118]</sup>. In addition, a recent study revealed that IAP KO mice have higher intestinal Ca<sup>2+</sup> uptake, which correlates with better biomechanical properties of trabecular bone<sup>[119]</sup>.

It has also been suggested that CaSR, abundantly expressed in apical and basolateral membranes of enterocytes in humans, rats and mice<sup>[120-122]</sup> may also participate in the local regulation of intestinal Ca<sup>2+</sup> absorption. Intestinal CaSR - specific KO mice showed an altered intestinal integrity, disbalanced gut microbiota and a pro-inflammatory status<sup>[123-125]</sup>. Rodrat *et al*<sup>[101]</sup> have recently observed that high-dose of 1,25(OH)<sub>2</sub>D<sub>3</sub> or high concentration of luminal calcium reduced Ca<sup>2+</sup> transport across a Caco-2 monolayer. The authors proposed that CaSR would sense luminal calcium triggering a local inhibitory feedback mechanism to restrict excessive Ca<sup>2+</sup> uptake<sup>[101]</sup>. This inhibitory loop could possibly involve locally produced FGF-23, which has been observed to counteract the enhanced duodenal Ca<sup>2+</sup> transport in mice exposed to 1,25(OH)<sub>2</sub>D<sub>3</sub> for a long term<sup>[32,100]</sup>.

## INTESTINAL Ca<sup>2+</sup> ABSORPTION UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS

Intestinal Ca<sup>2+</sup> absorption changes according to the different physiological conditions. It is promoted under high Ca<sup>2+</sup> demands such as growth, pregnancy, lactation, dietary Ca<sup>2+</sup> deficiency and high physical activity. In contrast, the intestinal Ca<sup>2+</sup> transport decreases with aging.

### Growth

In small and premature infants, who need higher dietary calcium to have a positive balance, the intestinal Ca<sup>2+</sup> absorption occurs through a passive paracellular pathway. These infants are unable to upregulate the transcellular pathway<sup>[126]</sup>. A progressive declination in the predominance of the paracellular pathway has been observed from childhood to adulthood<sup>[127]</sup>. Similarly, in rodents the intestinal Ca<sup>2+</sup> absorption changes with age in order to reach the requirements for bone mineralization during growth. In suckling rat, the intestinal Ca<sup>2+</sup> transport occurs predominantly through paracellular pathway; at weaning a transition to saturable absorption occurs indicating a larger contribution by the transcellular pathway during development, and then the absorption goes back to the paracellular pathway in adulthood<sup>[128]</sup>. The molecular changes associated with these alterations in the intestinal Ca<sup>2+</sup> absorption is not well elucidated. With respect to the transcellular movement, the mRNA expressions of



*Trpv6*, *Cabp9K*, and *Pmca1* in the duodenum have been first noted at 14 d and peaked at 21 days in rodents<sup>[129]</sup>. Akhter *et al*<sup>[130]</sup> have demonstrated at 6 weeks of age that *Cabp9K* was highly expressed in the duodenum with a small amount in the jejunum and cecum, and at 44 wk it was no longer detected in jejunum and cecum, but remained in the duodenum. With regard to paracellular pathway, Holmes *et al*<sup>[131]</sup> have demonstrated in the mice jejunum a decrease in *Cldn2* and increases in *Cldn12* and *Cldn15* with age. These studies highlight that the levels of molecules involved in the intestinal Ca<sup>2+</sup> absorption are not constant throughout growth. The potential modulators of changes in the intestinal Ca<sup>2+</sup> absorption during postnatal development are calcitriol, PRL and milk lactose<sup>[132]</sup>.

### Pregnancy

One of the physiological changes in pregnancy involves alterations in Ca<sup>2+</sup> metabolism. The adequate growth and development of the fetus is associated with increased intestinal Ca<sup>2+</sup> absorption and renal reabsorption in the mother. In the first trimester of human pregnancy, the intestinal Ca<sup>2+</sup> absorption increases twofold being maintained this increment to term. This doubling in the intestinal Ca<sup>2+</sup> absorption seems to be the major maternal adaptation to reach the fetal requirements for Ca<sup>2+</sup> either in humans or in rodents<sup>[133]</sup>. The classical calcitropic hormones as calcitriol, PTH and CT seem not to be main responsible for the Ca<sup>2+</sup> demand during pregnancy and lactation<sup>[134]</sup>. The intestinal Ca<sup>2+</sup> absorption has been shown to be VDR-independent in pregnant VDR knockout mice<sup>[135]</sup>. Pregnancy up-regulates intestinal Ca<sup>2+</sup> absorption and skeletal mineralization independently of the vitamin D receptor. It has been suggested that other hormones such as PRL, placental lactogen and GH or other factors could contribute to the doubling of intestinal Ca<sup>2+</sup> absorption in normal pregnancy<sup>[133]</sup>.

### Lactation

After birth, the maternal mammalian gland secretes an elevated amount of Ca<sup>2+</sup>, which could reach up to 1000 mg/day of milk Ca<sup>2+</sup>. In order to provide an extra Ca<sup>2+</sup> for milk production during lactating period, the osteoclast-mediated bone resorption and the intestinal Ca<sup>2+</sup> absorption is increased. The hormone responsible for milk calcium secretion in the stage of lactation remains uncertain, but there is some evidence that the lactogenic hormone PRL regulates that process. Charoenphandhu *et al*<sup>[134]</sup> have proposed that PRL stimulates the intestinal Ca<sup>2+</sup> absorption in a two-step manner. In step 1, PRL increases the baseline of intestinal Ca<sup>2+</sup> absorption in lactating rats through an increment in the TRPV6 mRNA, whereas in step 2, the suckling-induced PRL could induce further increased intestinal Ca<sup>2+</sup> absorption twofold over the newly increased baseline. High levels of PRL (400-800 mg/mL) are required to induce an acute enhancement in the intestinal Ca<sup>2+</sup> absorption, which is not attained without suckling<sup>[136]</sup>. The suckling-increased Ca<sup>2+</sup> transport in rats occurs either in the small intestine or the large intestine. This increment peaks after 30 min of suckling and lasts for 30-45 min post-suckling<sup>[12]</sup>. Wongdee *et al*<sup>[97]</sup> have demonstrated that PRL upregulated the expression of TRPV6 and PMCA<sub>1b</sub> in the duodenum of lactating rats. In addition, they have observed upregulation of FGF-23 protein expression in the duodenum and cecum of the same animals. They interpreted that PRL was responsible for the intestinal adaptation induced by lactation, which was compensated with an increase in FGF-23 to prevent excessive Ca<sup>2+</sup> absorption that might be harmful to lactating rats.

The role of calcitriol in the hyperabsorption of Ca<sup>2+</sup> during lactation is not clear. Kovacs<sup>[137]</sup> has reported that preterm and term babies absorb Ca<sup>2+</sup> through a passive non saturable process, which is facilitated by the lactose content in the milk. As they mature, they begin to absorb *via* a saturable calcitriol-dependent mechanism. This explains why vitamin D-deficient rickets appears much later, six to 18 mo after birth<sup>[138]</sup>. The phenotype of poor weight gain and low BMD in mice with a nonfunctional VDR is only observed after weaning, which indicates that intestinal Ca<sup>2+</sup> absorption is not calcitriol-dependent while suckling<sup>[132]</sup>. Recently, Zhang *et al*<sup>[139]</sup> have demonstrated the effects of maternal 25(OH) D<sub>3</sub> administration during lactation on sows and piglets. The intestinal Ca<sup>2+</sup> absorption was higher in treated sows as compared to non-treated sows, which are attributed to increased mRNA expressions of renal CYP27B1 and duodenal VDR, TRPV6, and CaBP-D9k. The piglets suckling sows receiving 25(OH) D<sub>3</sub> exhibited higher Ca<sup>2+</sup> content in tibia and femur; these effects were associated with higher plasma levels of calcitriol, which increased the gene expression of proteins involved in the intestinal Ca<sup>2+</sup> transport, *e.g.*, VDR and *Cldn-2* in ileum and VDR and CB<sub>9k</sub> in colon. In other words, 25(OH)D<sub>3</sub> supplementation during lactation improved bone health of both sow and piglet.

The temporal loss of bone mass during lactation is recovered promptly by mechanisms not quite clear. The bone health of the mother could be slightly or

severely compromised leading to fragility fractures in some women. Full recovery of calcium content and bone strength is not always achieved after weaning. Nevertheless, changes in calcium and bone metabolism during pregnancy and lactation in most women are normal, transient and without deleterious effects in the long-term<sup>[140]</sup>.

### Aging

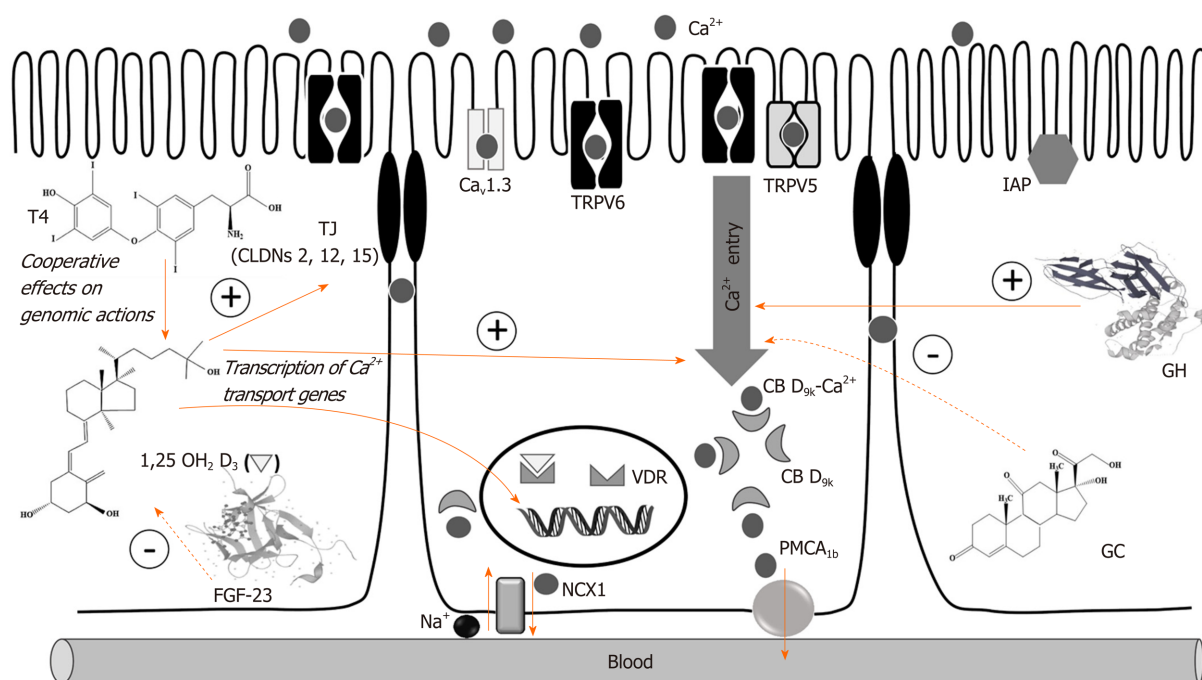
As a consequence of aging, the intestinal Ca<sup>2+</sup> absorption decreases either in humans or in rodents. In humans, malabsorption of Ca<sup>2+</sup> begins approximately at between 65 and 70 years<sup>[141]</sup>. In postmenopausal women, this deterioration begins earlier, but is reversible with estrogen therapy<sup>[142]</sup>. Among the different reasons for this decrease related to vitamin D metabolism could be mentioned: (1) Decreased renal synthesis of calcitriol by aged kidney; (2) Intestinal resistance to circulating calcitriol; (3) Decreased intestinal VDR; (4) Decreased skin synthesis of vitamin D; and (5) Substrate deficiency of vitamin D<sup>[143]</sup>. Song *et al*<sup>[144]</sup> have demonstrated that low levels of VDR in mice heterozygous for the VDR gene KO cause resistance of intestinal Ca<sup>2+</sup> absorption to calcitriol. This resistance appears to be generated by the low translation of CB<sub>9k</sub>, which occurs after binding VDR with its ligand. Ramsubeik *et al*<sup>[142]</sup> have reported that beyond the traditional focus on Ca<sup>2+</sup> and vitamin D, some other factors also influence intestinal Ca<sup>2+</sup> absorption in post-menopausal women such as dietary intake of kilocalories, carbohydrates, and potassium. See **Figure 1** for details about the molecular mechanisms involved in the intestinal Ca<sup>2+</sup> absorption and the regulation by hormones.

## OXIDATIVE STRESS AND ANTIOXIDANT AGENTS IN THE REGULATION OF INTESTINAL CALCIUM ABSORPTION

Reactive oxygen species (ROS) are by-products of normal cellular metabolism and there are enzymatic and non-enzymatic defense-systems in charge of maintaining a balance between ROS production and depletion. When this equilibrium fails, it is due to overproduction of ROS or to a deficiency in protective responses, oxidative stress arises, which can alter lipids, proteins and nucleic acids, thus provoking cell dysfunction and tissue damage. Gastrointestinal tract is an important source of ROS. Despite the protective barrier provided by intestinal mucosa and its adequately-distributed microbiota, digestion-endproducts and pathogens can trigger inflammatory response which favors oxidative stress. Consequently, various gastrointestinal pathologies such as gastroduodenal ulcers, cancer and inflammatory bowel disease are associated with oxidative stress<sup>[145,146]</sup>.

It has been demonstrated that intestinal Ca<sup>2+</sup> absorption is also affected by oxidative stress. A study carried out in our laboratory demonstrated that DL-buthionine-S, R-sulfoximine (BSO) reduced intestinal Ca<sup>2+</sup> absorption in rachitic chicks treated with cholecalciferol. BSO is an aminoacid-analogue which inhibits the synthesis of glutathione (GSH), one of the most important non-enzymatic intestinal antioxidant. The gut redox status was restored after intraluminal addition of GSH and intestinal Ca<sup>2+</sup> absorption returned to baseline<sup>[147]</sup>. BSO also reduced the activity of IAP, an enzyme presumed to play a role in Ca<sup>2+</sup> absorption, which was affected by the overabundance of ROS triggered by BSO. This reinforces the idea about the potential inhibitory effect of oxidative stress on the intestinal absorption of the cation<sup>[148]</sup>.

Posterior studies with different pro-oxidant drugs gave more information about the inhibition of Ca<sup>2+</sup> absorption by ROS overproduction. Such is the case of menadione (MEN), a synthetic precursor of vitamin K used in anti-cancer therapy. MEN metabolism starts by one-electron reduction and originates unstable semiquinone-radicals which rapidly react with O<sub>2</sub>. As a result, the semiquinone-radical cycles back to MEN and .O<sub>2</sub><sup>-</sup> is generated, which becomes H<sub>2</sub>O<sub>2</sub> through spontaneous or enzymatic dismutation<sup>[149]</sup>. Since GSH acts as an electron donor, intestinal administration of MEN (2.5 µmol /kg b. w.) depletes this antioxidant tripeptide triggering oxidative stress and diminishing the enzymatic activity of IAP and PMCA<sub>1b</sub><sup>[150]</sup>. In the same direction, Areco *et al*<sup>[151]</sup> have observed that intraperitoneal MEN was also able to reduce the expression of PMCA<sub>1b</sub>, CB<sub>28K</sub> and CLDN 2 in the intestinal mucosa of chicks treated with the quinone. This would contribute to explaining the transient reduction in Ca<sup>2+</sup> absorption caused by MEN, which appeared 30 minutes after treatment and lasted for less than ten hours. It is worth noting that this pro-oxidant also provoked apoptosis of enterocytes, thus determining the loss of approximately 30% of absorptive epithelial cells. The apoptotic process involved both the intrinsic and extrinsic pathways. An initial mitochondrial GSH depletion produced a reduction in mitochondrial membrane potential followed by the release of



**Figure 1 Effects of hormones on intestinal  $\text{Ca}^{2+}$  absorption.** Calcitriol stimulates the transcellular and paracellular absorptive pathways by inducing the expression of genes and proteins involved in  $\text{Ca}^{2+}$  transport and modifying the permeability of tight junctions. Thyroid hormones enhance the genomic actions of calcitriol whereas glucocorticoids inhibit the transcellular pathway by affecting the expression of  $\text{Ca}^{2+}$  transporting proteins. Fibroblast growth factor inhibits the intestinal  $\text{Ca}^{2+}$  absorption antagonizing  $1,25(\text{OH})_2\text{D}_3$  action. Growth hormone enhances the intestinal  $\text{Ca}^{2+}$  absorption through vitamin D dependent and independent mechanisms.  $\text{PMCA}_{1b}$ : Plasma membrane  $\text{Ca}^{2+}$ -ATPase;  $\text{CB}_{9k}$ : Calbindin 9k;  $\text{Ca}_v1.3$ :  $\text{Ca}^{2+}$  channel voltage-dependent L type  $\alpha 1D$  subunit; TRPV5: Transient receptor potential vanilloid 5; TRPV6: Transient receptor potential vanilloid 6; CLDNs 2, 12 and 15: Claudins 2, 12 and 15; NCX1:  $\text{Na}^+/\text{Ca}^{2+}$  exchanger;  $1,25(\text{OH})_2\text{D}_3$ : Calcitriol; FGF-23: Fibroblast growth factor; GH: Growth hormone; GC: Glucocorticoids; T4: Thyroxine; TJ: Tight junction; VDR: Vitamin D receptor.

cytochrome c into the cytoplasm and DNA fragmentation (intrinsic apoptotic pathway). Mitochondrial dysfunction induced by MEN affected Krebs-cycle only partially, since it reduced the activity of malate dehydrogenase in 18% and the one of  $\alpha$ -ketoglutarate dehydrogenase in 30% [152]. Extrinsic apoptotic pathway was also favored by MEN, which was evidenced by the expression of FAS, FASL and caspase-3 [153]. Interestingly, quercetin, an anti-inflammatory and anti-apoptotic flavonoid with important protective properties in the intestine [154], could reverse the inhibitory effect of MEN. This flavonoid blocked the alterations in the mitochondria membrane potential triggered by MEN, thus blocking the apoptotic route dependent on FAS/FASL-caspase 3. This anti-apoptotic effect, based on the capacity of quercetin to preserve GSH levels, contributed to maintaining the absorptive enterocytes functioning [153].

The inhibitory effect of MEN on  $\text{Ca}^{2+}$  movement can also be prevented or restored by some protective drugs such as glutamine [155,156], an anti-inflammatory and anti-apoptotic drug associated with diverse functions of intestinal mucosa such as growth and reparation [157,158]. Moine *et al* [155,156] have observed that glutamine normalized the content of different molecules involved in both calcium absorption pathways as well as the levels of GSH and the activity of antioxidant enzymes.

Similarly, a monodosis of 10 mg/kg b. w. of melatonin (MEL), a pineal hormone also secreted in the gastrointestinal tract, has been shown to restore  $\text{Ca}^{2+}$  absorption previously reduced by MEN. This effect was the result of the normalization of the activity of antioxidant enzymes superoxide dismutase and catalase and the restoration of  $\cdot\text{O}_2^-$  levels to basal status. MEL also restored the expression of proteins involved in both  $\text{Ca}^{2+}$  absorptive pathways [151]. These protective properties of MEL were reinforced in various studies that revealed anti-inflammatory effects of MEL in the intestine [159], helping to maintain epithelial integrity and digestive function [160], and reducing the risk of cancer [161].

Sodium deoxycholate (NaDOC) is a bile salt that depletes GSH, exerting a similar effect to the one of MEN. Rivoira *et al* [162] have demonstrated that high physiological doses of this salt inhibit intestinal  $\text{Ca}^{2+}$  absorption in a time and dose-dependent fashion. NaDOC mainly affects the transcellular pathway since it inhibits the expression of  $\text{PMCA}_{1b}$ ,  $\text{CB}_{D_{28k}}$  and NCX1. In addition, this bile-salt generates ROS and mitochondrial changes which eventually lead to apoptosis [162]. However, there are some bile acids that are able to preserve  $\text{Ca}^{2+}$  absorption. Lithocholic (LCA) is a

secondary bile acid that binds to VDR and acts as an endogenous agonist of vitamin D<sup>[163]</sup>, which has been proposed as a potential antitumoral agent<sup>[164]</sup>. LCA has proved to normalize the expression of genes and proteins involved in the transcellular pathway of Ca<sup>2+</sup> absorption affected by NaDOC and restore oxidative stress parameters such as .O<sub>2</sub><sup>-</sup> and the levels of protein carbonyl groups. This acid also attenuates the increase in the permeability of mitochondrial membrane triggered by NaDOC, being able to block the apoptosis induced by NaDOC when co-administered intraluminally with this drug. As a result, LCA avoids the reduction in the transcellular Ca<sup>2+</sup> absorption provoked by NaDOC<sup>[165]</sup>.

There are also pathological conditions that can favor oxidative stress and consequently lead to a reduction in the intestinal Ca<sup>2+</sup> absorption. One of these conditions is Type-1 diabetes mellitus (T1DM). Rivoira *et al*<sup>[166]</sup> have shown that diabetic Wistar rats induced by the injection of streptozotocin (STZ), absorbed less Ca<sup>2+</sup> than the control group. This reduction was transitory and reversible by insulin treatment. Interestingly, STZ-induced diabetes produced an overexpression of the proteins involved in the transcellular pathway which returned to basal levels after 60 d. This initial increase in the expression of NCX1, PMCA<sub>1b</sub> and TRPV6 has been interpreted as a possible compensatory effect to counteract the reduction in Ca<sup>2+</sup> absorption, probably associated with an imbalanced redox status.

Ca<sup>2+</sup> transport across intestinal epithelium is also impaired in experimental metabolic syndrome. Rodriguez *et al*<sup>[167]</sup> have observed that animals with a fructose-rich diet presented alterations in intestinal redox status, which were evidenced by a marked increase in .O<sub>2</sub><sup>-</sup>, lower activity of antioxidant enzymes and a reduction in GSH. These animals also had nitrosative stress with increased nitric oxide and higher nitrotyrosine content of proteins. This global redox disequilibrium determined a combined alteration of both trans and paracellular pathways of Ca<sup>2+</sup> absorption that might have been aggravated by a pro-inflammatory state with increased IL-6 and NF-κB. It is noteworthy that a subcutaneous injection of naringin (40 mg/kg b. w. during 4 wk on a daily basis), an antioxidant flavonoid present in grape fruit and other citrics, duplicated GSH level and blocked both inflammation and redox unbalance triggered by fructose-rich diet, consequently protecting intestinal Ca<sup>2+</sup> absorption<sup>[167]</sup>. These findings go in line with similar antioxidant effects of naringin in ischemia-reperfusion models<sup>[168]</sup>. The anti-inflammatory potential of naringin on intestinal mucosa has been confirmed by different studies in ulcerative colitis<sup>[169]</sup>, sepsis-induced intestinal injury<sup>[170]</sup>, and gastrointestinal tumorigenesis<sup>[171]</sup>. It has also been found that naringin contributes to maintaining an equilibrium between the different components of the microbiota<sup>[172]</sup>, thus preventing dysbiotic processes that could lead to ROS overproduction and indirectly affect Ca<sup>2+</sup> absorption<sup>[173]</sup>.

**Table 1** shows the influence of prooxidant conditions on the intestinal Ca<sup>2+</sup> absorption and the reversal/protection by antioxidants.

## INTESTINAL Ca<sup>2+</sup> ABSORPTION UNDER PATHOLOGICAL ALTERATIONS

### *Inflammatory bowel diseases*

Crohn's disease and ulcerative colitis, the main forms of inflammatory bowel disease, are characterized by chronic inflammation of the intestine that can deteriorate the intestinal Ca<sup>2+</sup> absorption<sup>[174]</sup>. In patients with Crohn's disease, this alteration may be due to vitamin D deficiency, magnesium deficiency, excessive use of glucocorticoids and/or intestinal resection. Vitamin D treatment has been shown to improve the intestinal Ca<sup>2+</sup> absorption in these patients<sup>[175]</sup>.

### *Celiac disease*

Patients with celiac disease (CD) frequently present Ca<sup>2+</sup> deficiency, low BMD and metabolic bone diseases. In children and adolescents with CD, the Ca<sup>2+</sup> deficiency may produce growth alterations and difficulties in peak bone mass achievement<sup>[176]</sup>. In the elderly, Ca<sup>2+</sup> deficiency leads to low BMD and increased fracture risk<sup>[177]</sup>. Bone alterations partially result from impaired intestinal Ca<sup>2+</sup> absorption due principally to the loss of villous cells in the duodenum, where the active Ca<sup>2+</sup> transport occurs<sup>[178]</sup>. Steatorrhea, vitamin D deficit and changes in the mechanisms of Ca<sup>2+</sup> absorption are other factors that contribute to the Ca<sup>2+</sup> malabsorption<sup>[179]</sup>. Hypovitaminosis D in CD patients is also a consequence of its malabsorption and the intestinal mucosal lesion<sup>[180]</sup>, which lead to reduce the plasma calcitriol levels, and therefore, the intestinal Ca<sup>2+</sup> absorption.

### *Morbid obesity and bariatric surgery*



**Table 1** Effects of pro-oxidant conditions on intestinal Ca<sup>2+</sup> absorption and associated parameters

Pro-oxidant condition	Effects on genes and proteins involved in intestinal Ca <sup>2+</sup> absorption	Effect on REDOX state	Effects of antioxidant/ protective molecules	Effects on apoptosis
BSO <sup>[147,148]</sup>	Inhibition of IAP activity	Decrease in GSH content	GSH administration normalized intestinal Ca <sup>2+</sup> absorption	Not evaluated
MEN <sup>[149-156]</sup>	Decrease in PMCA <sub>1b</sub> gene-protein expression and activity. Decrease in CB D <sub>28k</sub> and CLDN 2 gene-protein expression	Depletion of GSH content; Increase in ROS and protein carbonyls; Enhancement in SOD and CAT activity	QT, MEL and GLT administration normalized intestinal Ca <sup>2+</sup> absorption and associated parameters	Activation of intrinsic and extrinsic pathways
NaDOC <sup>[162,165]</sup>	Decrease in PMCA <sub>1b</sub> mRNA. Inhibition of PMCA <sub>1b</sub> , CB D <sub>28k</sub> and NCX1 protein expression	Depletion of GSH content; Increase in ROS and activity of SOD, CAT and GPx; Increase in iNOS protein expression and NO <sup>•</sup> content	QT and UDCA administration avoided the inhibition of intestinal Ca <sup>2+</sup> absorption caused by NaDOC	Activation of intrinsic and extrinsic pathway
Diabetes <sup>[166]</sup>	Enhancement in expression of NCX1, PMCA <sub>1b</sub> and TRPV6 proteins and CLDN 2 gene expression	Decrease in GSH content; Increase in SOD activity and ROS levels	Insulin treatment restored redox state and intestinal Ca <sup>2+</sup> absorption	Not evaluated
Metabolic syndrome <sup>[167]</sup>	Decrease in TRPV6, PMCA <sub>1b</sub> , CB D <sub>9k</sub> , CLDN 2, CLDN 12 and VDR protein expression; Decrease in IAP activity	Enhancement in protein carbonyls, NO <sup>•</sup> levels and nitrotyrosine content in proteins; Decrease in SOD and CAT activity	Administration of NAR prevented the reduction of intestinal Ca <sup>2+</sup> absorption caused by fructose-rich diet	Not evaluated

BSO: DL-buthionine-S, R-sulfoximine; CAT: Catalase; CB D<sub>28k</sub>: Calbindin D<sub>28k</sub>; CB D<sub>9k</sub>: Calbindin D<sub>9k</sub>; CLDN 2: Claudin 2; CLDN 12: Claudin 12; GLT: Glutamine; GPx: Glutathione peroxidase; GSH: Glutathione; AP: Alkaline phosphatase; MEL: Melatonin; MEN: Menadione; NaDOC: Sodium deoxycholate; NAR: Naringin; NCX1: Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; NO<sup>•</sup>: Nitric oxide; PMCA<sub>1b</sub>: Plasma membrane Ca<sup>2+</sup>ATPase; QT: Quercetin; SOD: Superoxide dismutase; TRPV6: Transient receptor potential vanilloid type 6; UDCA: Ursodeoxycholic acid; VDR: Vitamin D receptor.

Bariatric surgery (BS) is a valuable option to treat the morbid obesity. However, these procedures may produce a decrease in the BMD increasing the risk of bone fractures, particularly when the duodenum is bypassed, as occurs in Roux-en-Y Gastric Bypass (RYGB). Since the duodenum is the site where the active transport of Ca<sup>2+</sup> occurs<sup>[181]</sup>, the RYGB contributes to decreasing not only the intestinal Ca<sup>2+</sup> absorption but also the absorption of other mineral and nutrients. The RYGB prevents the active calcitriol-mediated Ca<sup>2+</sup> transcellular pathway in the duodenum and proximal jejunum, which resembles in certain extension a proximal intestine-specific *Vdr* KO animal model<sup>[182]</sup>. Furthermore, hypovitaminosis D is common in patients exposed to BS, which seems to be multifactorial, some factors being related to obesity and others related to the type of the surgical procedure and its consequences<sup>[183,184]</sup>. Indeed, the vitamin D deficiency would contribute to inhibiting the intestinal Ca<sup>2+</sup> absorption, leading to impaired Ca<sup>2+</sup> homeostasis and bone density. The reduction in the intestinal Ca<sup>2+</sup> absorption<sup>[185]</sup> produces secondary hyperparathyroidism, and ultimately triggers bone loss<sup>[186]</sup>. Vitamin D supplementation after RYGB has not always avoided a decrease in BMD, suggesting that other factors should be involved in the bone loss<sup>[132]</sup>. In the Sleeve Gastrectomy, another very common procedure of BS, the contact time between Ca<sup>2+</sup> and intestinal cells is shortened and, hence, the intestinal Ca<sup>2+</sup> absorption decreases.

### Diabetes

The intestinal Ca<sup>2+</sup> absorption in diabetes has been poorly addressed. Most studies have dealt with experimental diabetes provoked by alloxan or STZ. Schneider *et al*<sup>[187]</sup> have demonstrated that the intestinal Ca<sup>2+</sup> absorption is decreased in rats made diabetic with alloxan, which is due to a decrease in the lumen-to-plasma Ca<sup>2+</sup> flux in the duodenum and ileum. A reduction in intestinal Ca<sup>2+</sup> absorption has been reported to be associated with low circulating levels of calcitriol, decreased VDR number and CB<sub>9k</sub> content in diabetic rats<sup>[188,189]</sup>. In our laboratory we have also observed that the intestinal Ca<sup>2+</sup> absorption decreases by insulin deficiency in rats injected with STZ, a model of T1DM. However, the effect was relatively rapid and transient leading to a time dependent adaptation, returning the intestinal Ca<sup>2+</sup> absorption to normal values. The inhibition was accompanied by redox changes that produce oxidative stress, which may lead to alterations in the duodenum permeability. Both the redox state of the intestine and the intestinal Ca<sup>2+</sup> absorption were normalized after insulin administration, which was independent of vitamin D status<sup>[166]</sup>. In a clinical study with adolescent girls using a dual-stable isotope approach to evaluate Ca<sup>2+</sup> absorption,

Weber *et al*<sup>[190]</sup> did not find adverse effects of T1DM on gastrointestinal Ca<sup>2+</sup> absorption. Since T1DM is characterized by bone loss, alteration in bone remodeling, low BMD and increased risk of fractures<sup>[191]</sup>, further investigation should be done in order to clarify whether a reduction in intestinal Ca<sup>2+</sup> absorption contributes to development of bone disease associated to insulin deficiency.

### Hypercalciurias

Hypercalciuria is very common in patients with kidney stones. Although the molecular mechanisms underlying hypercalciuria are not well elucidated, it is considered that increased intestinal Ca<sup>2+</sup> absorption contributes to the pathogenesis<sup>[192]</sup>. The idiopathic hypercalciuria is the most common form, but it has a polygenic trait, which makes more difficult to understand the pathogenesis. In the Dent disease, a monogenic disorder associated with hypercalciuria, it has also been found Ca<sup>2+</sup> hyperabsorption. Wu *et al*<sup>[193]</sup> have demonstrated that the disruption of PI(4,5)P<sub>2</sub> 5-phosphatase activity by Dent-causing mutations of OCRL gene may explain the increased intestinal Ca<sup>2+</sup> absorption. The authors conclude that the TRPV6 activity is enhanced due to the increased transcription of TRPV6 gene provoked by increased calcitriol<sup>[194]</sup> and/or release of TRPV6 suppression under Dent conditions.

The hereditary hypophosphatemic rickets with hypercalciuria is a rare autosomal recessive disorder with a prevalence of 1:250000. The patients carry loss-of-function mutations in the sodium-phosphate co-transporter NaPi-2c, which cause an increase in the urinary Pi excretion, hypophosphatemia, bowing, short stature and elevated calcitriol levels. Consequently, the intestinal Ca<sup>2+</sup> absorption increases, the PTH dependent Ca<sup>2+</sup>-reabsorption in the distal renal tubules decreases, resulting in hypercalcemia, which leads to nephrocalcinosis in half of patients<sup>[195]</sup>.

### Turner syndrome

Women with Turner syndrome have an increased risk of osteoporosis mainly due to inadequately treated primary ovarian insufficiency and intrinsic bone abnormalities. These patients usually present some comorbid conditions that may further increase the risk of osteoporosis, such as vitamin D deficiency, CD and inflammatory bowel disease<sup>[196]</sup>. All these conditions may be associated with deficient Ca<sup>2+</sup> absorption *via* different mechanisms such as endocrine deregulation of Ca<sup>2+</sup> metabolism or oxidative stress among others.

### Thalassemia

Osteoporosis and impairment of Ca<sup>2+</sup> homeostasis are frequent complications of thalassemia. Studies in thalassemic patients and animal models suggested that a defective Ca<sup>2+</sup> absorption might be a cause of thalassemic bone disorder. The possible mechanisms associated with intestinal Ca<sup>2+</sup> malabsorption in thalassemia are alterations in the Ca<sup>2+</sup> transporters and hormonal controls of the transcellular and paracellular intestinal transport systems<sup>[108]</sup>.

## CONCLUSION

There has been an important progress in molecular studies related to the effects of calcitriol on intestinal Ca<sup>2+</sup> absorption in rodents and birds. However, information about the role of other hormones and dietary factors are scarce. The lack of information about the molecular alterations in the intestinal Ca<sup>2+</sup> absorption that accompany human pathologies is even greater. Since oxidative stress has shown to produce a powerful influence on the intestinal Ca<sup>2+</sup> absorption and the prevention or restoration by antioxidants in experimental animals have proved to be very successful, it would be worth investigating these aspects in humans carrying pathologies associated with altered intestinal Ca<sup>2+</sup> absorption.

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