ABSTRACT

Extracellular calcium participates in several key physiological functions, such as control of blood coagulation, bone calcification or muscle contraction. Calcium homeostasis in humans is regulated in part by genetic factors, as illustrated by rare monogenic diseases characterized by hypo or hypercalcaemia. Both serum calcium and urinary calcium excretion are heritable continuous traits in humans. Serum calcium levels are tightly regulated by two main hormonal systems, i.e. parathyroid hormone and vitamin D, which are themselves also influenced by genetic factors. Recent technological advances in molecular biology allow for the screening of the human genome at an unprecedented level of detail and using hypothesis-free approaches, such as genome-wide association studies (GWAS). GWAS identified novel loci for calcium-related phenotypes (i.e. serum calcium and 25-OH vitamin D) that shed new light on the biology of calcium in humans. The substantial overlap (i.e. CYP24A1, CASR, GATA3; CYP2R1) between genes involved in rare monogenic diseases and genes located within loci identified in GWAS suggests a genetic and phenotypic continuum between monogenic diseases of calcium homeostasis and slight disturbances of calcium homeostasis in the general population. Future studies using whole-exome and whole-genome sequencing will further advance our understanding of the genetic architecture of calcium homeostasis in humans. These findings will likely provide new insight into the complex mechanisms involved in calcium homeostasis and hopefully lead to novel preventive and therapeutic approaches.

Keyword: calcium, monogenic, genome-wide association studies, genetics

INTRODUCTION: CONTROL OF CALCIUM HOMEOSTASIS

Extracellular calcium participates in several key physiological functions, such as control of blood coagulation, bone calcification or muscle contraction. Even if important calcium fluxes are exchanged between organs implicated in the import, storage and export in and out of the organism (the intestine, bone and kidneys), serum calcium concentration is kept stable, with variations that do not exceed 2–3% and that are mainly due to circadian rhythmicity [1]. However, even with a thorough control of its concentration, calcium reaches saturation levels in the plasma and only strong inhibitors, e.g. magnesium, fetuin A or albumin, can prevent precipitation and stabilize calciprotein particles [2]. Although calcium has crucial physiological roles, it can do harm if deposited inappropriately.

Regulation of extracellular calcium concentration and bio-mineralization depends upon a complex network of hormones and intricate feedback loops. Like sodium homeostasis, which is regulated by both, a short-term-acting and G-protein-coupled receptor (GPCR)-dependent system (angiotensin 2) and a long-term-acting and nuclear receptor-dependent hormone (aldosterone), calcium homeostasis is maintained mainly by two systems, the parathyroid hormone (PTH) and vitamin D.

PTH

Biologically active PTH is a peptide that interacts, with high affinity, with PTH receptor 1 (PTH1R), triggering downstream intracellular signalling. PTH is synthetized as a pre-prohormone...
in the parathyroid glands and is secreted in a pulsatile manner, with infradian and circadian rhythms, in the blood stream as a 1–84 fragment (intact PTH). The half-life of biologically active PTH in the circulation is only a few minutes before it is degraded in a variety of smaller processed fragments with largely unknown functions [3, 4]. Recent data indicate that oxidation of PTH in certain clinical circumstances such as renal insufficiency could decrease the affinity of PTH for its receptor [5]. Parathyroid glands exert two major functions regarding calcium: sensing plasma calcium concentration—free-ionized calcium only—and adapting secretion of PTH. Once released in the bloodstream, PTH augments serum calcium levels directly by increasing calcium reabsorption by the kidney, calcium release by the bone and indirectly by increasing intestinal calcium uptake.

**CALCIUM-SENSING RECEPTOR**

In contrast to other ions for which it has been postulated that sensors exist but were never discovered, the identification of the calcium-sensing receptor (CASR) by Brown and Hebert was instrumental in deciphering a main passive regulator of serum calcium concentration [6]. First, by its expression in the parathyroid gland, CASR directly connects calcium sensing and control of PTH secretion and its downstream consequences. However, the effects of the CASR on calcium homeostasis extend beyond the parathyroid glands. Expressed in the intestine and the bone, CASR may regulate calcium fluxes in these organs even though its precise role is not fully established [7, 8]. In contrast, in the kidney, CASR has been shown to control serum calcium concentration independently of its effect on PTH secretion. Indeed, using double CASR–PTH knockout mice, Kantham et al. [9] showed that CASR defends against hypercalcaemia independently from PTH. Toka et al. [10] did provide additional evidence consistent with these results by studying CASR kidney-specific knockout mice. Finally, an elegant study by Loupy et al. [11] showed that CASR in the thick ascending limb regulates serum calcium concentration independently of PTH. Altogether, these studies displayed strong evidence that regulation of renal calcium excretion by CASR directly influences calcium homeostasis and identified CASR as a primary regulator of serum calcium concentration, independently of its effect on PTH secretion.

**VITAMIN D**

To fit with the high affinity in the ligand pocket of the nuclear receptor vitamin D receptor (VDR) and produce its effects, vitamin D needs to be activated by two hydroxylation steps, at position 25 and 1. It is not yet fully elucidated, which enzyme hydroxylates vitamin D at position 25 in the liver. One candidate is the mitochondrial cytochrome P450, subfamily 27, polypeptide 1 (CYP27A1), which is, however, mainly involved in cholesterol and bile acid metabolism and has high-capacity but only low-affinity 25-hydroxylase activity [12]. Since mice and humans with a CYP27A1 mutation exhibit normal 25-OH vitamin D concentrations, this enzyme probably plays a minor role in the transformation of vitamin D [13]. Another proposed enzyme involved in the 25-hydroxylation of vitamin D in the liver is the low-capacity, high-affinity microsomal cytochrome P450, subfamily 2R, polypeptide 1 (CYP2R1) [14]. CYP2R1 plays a crucial role in the metabolism of vitamin D, since patients and mice with homozygous mutations in this gene display low-circulating 25-OHD levels and rickets [15]. Surprisingly, double knockout mice for CYP2R1 and CYP27A1 exhibit normal levels of serum 25(OH) vitamin D, and no rickets, suggesting that another 25-hydroxylase exists and needs to be identified, at least in the mouse [16]. The second and highly regulated hydroxylation is much better characterized. CYP27B1 (1-alpha hydroxylase) is a highly regulated enzyme from the CYP450 family. Calcium, phosphate, PTH, calcitomin and the fibroblast growth factor 23 (FGF23)-Klotho axis are the main regulatory factors of CYP27B1, whereas 1,25(OH)2 vitamin D inhibits, in a negative feedback loop on CYP27B1, its own production. Vitamin D homeostasis depends, however, not only on production, but also on degradation, which is also highly regulated. Cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), hydroxylates 1,25(OH)2 vitamin D and targets it for further catabolism into calcitriolic acid and excretion. CYP24A1 is regulated, at the transcriptional level, in an antagonistic manner compared with CYP27B1, by calcium, phosphate, PTH, FGF23, Klotho and is activated by high levels of circulating 1,25(OH)2 vitamin D, thus inducing its own degradation. Active 1,25(OH)2 vitamin D stimulates intestinal calcium absorption, increases bone mineralization, but also bone resorption and favours calcium reabsorption by the kidney.

The main mechanisms of calcium homeostasis described here have been further explored at the molecular level by geneticists. This review now emphasizes how studies on rare monogenic diseases in patients bearing disturbances of calcium homeostasis allowed precise dissection of the molecular pathways involved in these pathologies. Moreover, we will show that hypothesis-free genome-wide association studies (GWAS) conducted in large population-based samples are powerful in detecting new genetic determinants of serum calcium and in validating known actors involved in the control of serum calcium levels. This review does not cover non-genetic causes of hyper or hypocalcaemia.

**GENETICS OF CALCIUM HOMEOSTASIS**

**Familial aggregation and heritability**

Both serum calcium and urinary calcium excretion have been shown to be heritable continuous traits in humans [17]. In 1747 pairs from the UK Adult Twin Registry, heritability was estimated to be 33% (95% CI: 21–45%) for serum calcium and 52% (95% CI: 41–61%) for 24-h urinary calcium excretion [17]. The unimodal distributions of serum calcium (symmetric and close to normal) and 24-h urinary calcium excretion (positively skewed, close to log-normal) in the general
population suggest that numerous genetic factors (i.e. polygenic traits) play a role in their control. The existence of rare monogenic diseases with disturbances in calcium homeostasis further underscores the importance of genetic factors. Similar to other phenotypes, it is likely that a genetic and phenotypic continuum exists between rare monogenic disorders of calcium homeostasis and genetic factors responsible for the control of calcium homeostasis in the general population (Figure 1). This continuum can be described as follows: (i) several mutations in a single gene can lead to the same monogenic disease (e.g. VDR, CASR [18]); (ii) different mutations in a single gene may lead to several monogenic disorders (e.g. CASR); (iii) although the genetic effect size within a monogenic context is usually large, other genes may modify the penetrance of the main mutation (e.g. CASR–PTH [19]); (iv) genes involved in rare monogenic diseases may play a role in common complex diseases and phenotypes (e.g. CASR, CYP24A1); (iv) a polygenic disorder/phenotype is considered to result from the addition of many small genetic effects, although one cannot exclude a mixture of big and small effects.

Genome-wide association studies

GWAS have made an important contribution to our understanding of the genetic determinants of human traits [20]. GWAS allow a hypothesis-free exploration of the entire human genome and identify genomic regions robustly associated with phenotypes of interest. Current DNA chips usually include 500,000 to 1 million single nucleotide polymorphisms (SNPs) to be genotyped at an affordable cost. During the past decade >2000, robust associations have been published in GWAS [20].

What are the principles underlying GWAS?

Once participants have been genotyped, several steps are conducted until a clean data set is obtained. In a second stage, additional genetic variants are being inferred using the linkage disequilibrium structure (so-called 'haplotype blocks') of the human genome and publicly available HapMap and/or 1000 Genomes data (so-called 'imputed SNPs'). First generation GWAS focused on common alleles (i.e. with a minor allele frequency usually >5%). More recent DNA chips now also allow the measuring of rarer genetic variants. The fact that GWAS focused on common variants does not imply that some of the association signals from GWAS do not arise from multiple rare variants. This results from the fact that a GWAS signal is not restricted to the so-called top SNP (i.e. SNPs with the lowest association P-value in a given region), but also includes the entire region. The length of the captured region strongly depends on the local linkage disequilibrium structure (i.e. correlation structure).

Once a specific region of the genome has been identified as being robustly associated with a phenotype of interest, a much additional work is needed to unravel the causal variant(s). Whenever a single gene is included in the associated region, this task is much easier than when dozens of genes are present. Also, the SNPs most significantly associated with the phenotype of interest are unlikely to be the true underlying causal variant.

When compared with candidate gene studies published before the GWAS era, GWAS findings are more robust in that they have to be replicated before the results can be published. As most GWAS associations correspond to small effect sizes, very large sample sizes are usually needed to replicate findings. GWAS are, therefore, less likely to generate false-positive findings than earlier candidate gene studies that were often underpowered and hard to replicate. Of note, the size of the effect from a GWAS signal provides limited information on the importance of the corresponding gene in the phenotype under study. SNPs available in genome-wide DNA arrays only represent a small subset of all SNPs throughout the human genome.

**Figure 1:** Genes involved in regulation of serum calcium concentration. Genes have been identified either by studies of monogenic diseases or by GWAS performed on serum calcium and on vitamin D. Genes represented in green are mainly involved in the PTH axis and those in red are mainly involved in the vitamin D axis. No GWAS has been conducted on PTH levels so far.
The first published GWAS for 25(OH)D was conducted in 4501 individuals of European descent and identified the rs2282679 near GC [group-specific component (vitamin D-binding protein)], rs3829251 near NADSYN1 (NAD synthetase 1)/DHCR7 (7-dehydrocholesterol reductase) and rs2060793 near CYP2R1 variants as being significantly associated with 25(OH)D levels [25]. In a later GWAS meta-analysis, three loci achieved genome-wide significance and replication for 25 (OH) vitamin D levels in 30,000 participants of European descent: (i) rs2282679 in GC; (ii) rs12785878 near NADSYN1/DHCR7 and (ii) rs10741657 near CYP2R1, whereas the CYP24A1 rs6013897 variant had genome-wide significance in the pooled sample [26]. These loci collectively explained between 1% and 4% of 25(OH) variance. GC is involved in vitamin D transport; DHCR7 is involved in the synthesis, and CYP2R1 in the hydroxylation, of cholesterol. Identification of CYP2R1 by GWAS for 25(OH)D strengthens its implication as the main 25-hydroxylase for vitamin D. A GWAS conducted in 572 Caucasian children with asthma did not identify additional variants associated with 25(OH)D [27]. So far variants identified in GWAS only explain a small proportion (~5%) of the heritability of 25(OH)D estimated by twin- and family studies [28]. Additional studies are needed to further decipher genetic determinants of 25(OH) vitamin D levels in humans.

A single GWAS, in 229 Hispanic Americans, was conducted for 1,25(OH)2D and no genome-wide significant signal was identified, yet 8 SNPs from a set of 50 selected SNPs were replicated for 1,25(OH)(2)D in a larger cohort: rs6680429, rs1348864, rs4559029, rs12667374, rs7781309, rs10505337, rs2486443 and rs2154175 [29]. Larger sample sizes are clearly needed to achieve sufficient power to detect and replicate signals across studies.

**Rare monogenic diseases involved in serum calcium control**

Genetic determinants of serum calcium levels are numerous and can be grouped into those affecting the CASR, PTH, vitamin D and other calcium regulatory axis. Since the pioneer work of Fuller Albright in the 1930s, large collections of rare conditions in which plasma calcium is disturbed either towards higher or lower levels have been identified, described, explored and categorized. With the emergence of molecular medicine, old unique entities have been subcategorized in a variety of conditions that can now be better explained and treated, e.g. familial hypocalciuric hypercalcaemia (FHII), previously thought to be a single condition which is now genetically heterogeneous, with at least three causative genes identified [CASR, GNA11 (guanine nucleotide-binding protein G protein), alpha 11], AP2SI (adaptor-related protein complex 2, sigma 1 subunit)]. If precise and complete clinical descriptions of patients are still mandatory, new powerful molecular tools have facilitated and accelerated the diagnosis and the discovery of genes causing orphan diseases. Some of the main rare diseases affecting calcium plasma levels are discussed hereafter (Table 1). Owing to space constraints, only a selection of conditions and publications is highlighted here and this review is neither systematic nor exhaustive.
In contrast, MENIN is a tumour suppressor protein involved in chromatin remodelling and expressed in several glandular tissues. When mutated, the coding gene MEN1 (multiple endocrine neoplasia syndrome type 1) leads to hypomorphic MENIN production that results in a complex MEN1. Primary hyperparathyroidism is one of the most frequently encountered complications of the syndrome, leading to classic hypercalcemia/hypophosphatemia with inappropriately high PTH levels.

PTH mediates its effect via PTHR1 which is a GPCR. Mutations in the genes coding for PTHR1 and for G protein alpha S (encoded by GNAS) that transduces the signal intracellularly, leads to peripheral resistance to PTH effects. This entity is called pseudo-hyperparathyroidism and leads to severe bone anomalies (chondrodysplasia of several types, including Eiken syndrome [35]) and renal resistance to PTH action. As GNAS is highly methylated, the phenotype depends on the parental inheritance of the mutated gene. A detailed description of the different subtypes of pseudo-hyperparathyroidism is beyond the scope of this review but can be found in Levine et al. [36].

### CASR-ASSOCIATED DISEASES

Human loss-of-function mutation in the CASR gene causes FHH when one allele is mutated and neonatal severe hyperparathyroidism (NSHPT) when both alleles are affected. FHH is characterized by decreased urinary calcium excretion and increased bone demineralization.

<table>
<thead>
<tr>
<th>Target</th>
<th>Gene</th>
<th>Mechanism</th>
<th>Disease</th>
<th>OMIM</th>
<th>Effect on plasma calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASR</td>
<td>CASR</td>
<td>Loss of function, Heterozygous</td>
<td>FHH type 1</td>
<td>145980</td>
<td>Hypercalcaemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Loss of function, Homozygous</td>
<td>NSHPT</td>
<td>239200</td>
<td>Hypercalcaemia</td>
</tr>
<tr>
<td>GNA11</td>
<td>Activating mutation</td>
<td>ADH type 1, including Bartter-like syndrome</td>
<td>601198</td>
<td>Hypocalcaemia</td>
<td></td>
</tr>
<tr>
<td>AP2S1</td>
<td>Loss of function</td>
<td>FHH type 2</td>
<td>145981</td>
<td>Hypercalcaemia</td>
<td></td>
</tr>
<tr>
<td>MEN1</td>
<td>Loss of Function</td>
<td>MEN type 1 with hyperparathyroidism</td>
<td>131100</td>
<td>Hypercalcaemia</td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>Loss of function</td>
<td>Familial isolated hyperparathyroidism</td>
<td>146200</td>
<td>Hypocalcaemia</td>
<td></td>
</tr>
<tr>
<td>GCM2</td>
<td>Loss of function</td>
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<td>146200</td>
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<tr>
<td>TBX1 and others (?)</td>
<td>Deletion chromosome 22</td>
<td>Di George, velocardiofacial, Catch22 syndrome</td>
<td>188400</td>
<td>Hypocalcaemia</td>
<td></td>
</tr>
<tr>
<td>FAM111A</td>
<td>Loss of function</td>
<td>Hypoparathyroidism and bone dysplasia</td>
<td>615292</td>
<td>Hypocalcaemia</td>
<td></td>
</tr>
<tr>
<td>GATA3</td>
<td>Loss of function</td>
<td>Hypoparathyroidism, renal dysplasia, sensorineural deafness</td>
<td>146255</td>
<td>Hypocalcaemia</td>
<td></td>
</tr>
<tr>
<td>GNAS</td>
<td>Loss of function, Pseudohypoparathyroidism</td>
<td>Chondrodysplasia</td>
<td>103580</td>
<td>Hypercalcaemia</td>
<td></td>
</tr>
<tr>
<td>PTHRI</td>
<td>Loss of function</td>
<td>Heterozygous</td>
<td>156400</td>
<td>Hypercalcaemia</td>
<td></td>
</tr>
<tr>
<td>Vitamin D</td>
<td>CYP27B1</td>
<td>Loss of function</td>
<td>Vitamin D-dependent rickets 1A</td>
<td>264700</td>
<td>Hypercalcaemia</td>
</tr>
<tr>
<td></td>
<td>CYP2RI</td>
<td>Loss of function</td>
<td>Vitamin D-dependent rickets 1B</td>
<td>600081</td>
<td>Hypercalcaemia</td>
</tr>
<tr>
<td></td>
<td>VDR</td>
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<td>277440</td>
<td>Hypercalcaemia</td>
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<tr>
<td></td>
<td>HNRNPC</td>
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<td>164020</td>
<td>Hypercalcaemia</td>
</tr>
<tr>
<td></td>
<td>CYP24A1</td>
<td>Loss of function</td>
<td>Infantile hypercalcaemia</td>
<td>143880</td>
<td>Hypercalcaemia</td>
</tr>
</tbody>
</table>

Secondary causes were omitted (e.g. hypomagnesaemia with secondary hypocalcaemia, hypophosphataemia...).
PTH-dependent hypercalcaemia, while NSHPT leads to severe early hyperparathyroidism and hypercalcaemia [37]. In mice in which CASR has been deleted, the severe phenotype mimicking human NSHPT could be rescued by suppressing PTH, as elegantly showed in experiments during which PTH and CASR were simultaneously inactivated [38]. The fact that these mice harbour only slightly elevated, but highly variable plasma calcium suggests that the regulation of serum calcium concentration and PTH by the CASR occurs independently. The hypocalciuria observed in CASR KO mice was not rescued in the double CASR–PTH knockout animals, suggesting that CASR in the kidney is necessary for normal calcium excretion by preventing excessive calcium reabsorption independently of PTH [39]. In contrast, gain-of-function mutations in the CASR gene observed in humans lead to autosomal-dominant hypocalcaemia type 1 (ADH1) [40]. Similarly, mice carrying an activating mutation in CASR exhibit hypocalcaemia, hypoparathyroidism, and hyperphosphataemia and decreased renal calcium reabsorption [41].

Further sequencing of CASR-negative FHH patients provided new insights into our understanding of the regulatory pathways of the CASR and identified genetic heterogeneity in this syndrome. FHH type 1 is the classical form due to CASR mutations. FHH type 2 is phenotypically similar, but results from loss-of-function mutations in a gene coding for the protein G alpha 11 (GNA11) [42]. This indicates that this protein G is implicated in the downstream intracellular signalling of CASR. Interestingly, activating mutations in the same gene could lead to the mirror image, i.e. ADH type 2, as seen with CASR. Finally, FHH type 3 patients, who have a different and more severe clinical course, including increased serum PTH concentrations, hypophosphataemia and osteomalacia were found to carry mutations in the gene coding for adaptor protein 2 sigma subunit (AP2S1), a protein involved in clathrin-mediated endocytosis [43]. This suggests that regulation of CASR endocytosis is critical for its normal function. This finding perfectly illustrates the fact that studying rare monogenic diseases may uncover new pathogenic mechanisms and in this case, identify new regulatory pathways for CASR and calcium homeostasis.

**CONDITIONS AFFECTING THE VITAMIN D AXIS**

In family-based studies, heritability estimates ranged from 20 to 80% for circulating levels of vitamin 25-OH-D(3) and from 30 to 48% for 1,25-OH(2)-D(3) levels [44, 45], which justifies the search for genetic determinants. In a twin study in Vietnam, heritability of 25(OH) vitamin D was high and significant in winter (70%), but not in summer, which suggests a predominant role of environmental conditions in summer [46].

Active 1,25(OH)₂ vitamin D level is the result of the fine regulation of production and degradation by cytochrome P450 enzymes. Genetic syndromes affecting production and degradation of 1,25 (OH)₂ vitamin D have been described and further subcategorized. Inactivating mutations in the CYP27B1 gene, coding for 1-alpha hydroxylase, lead to vitamin D-resistant rickets type 1a [47]. More debated is the role of CYP2R1 in vitamin D-resistant rickets type 1b, as discussed previously. Active 1,25(OH)₂ vitamin D produces its effect by its interaction with the VDR. Mutations in VDR provide severe peripheral resistance to the effect of 1,25(OH)₂ vitamin D and an autosomal

**FIGURE 2:** Tissue expression of genes involved in the regulation of serum calcium concentration. (A) Genes present in parathyroid glands. (B) Genes expressed in the bone. (C) Genes in the kidneys. PT, proximal tubule; TAL, thick ascending limb; CD, collecting duct.
CONCLUSION

Calcium homeostasis in humans is regulated in part by genetic factors, as illustrated by rare monogenic diseases. Serum calcium levels are tightly regulated by two main hormonal systems, i.e. PTH and vitamin D, which are themselves also influenced by genetic factors. Recent technological advances in molecular biology allow screening the human genome at an unprecedented level of detail and using hypothesis-free approaches, such as GWAS. GWAS identified novel loci for calcium-related phenotypes that shed new light on the biology of calcium in humans (Figure 2). There is likely a genetic and phenotypic continuum between rare monogenic diseases of calcium homeostasis and slight disturbances of calcium homeostasis in the general population. Future studies using whole-exome and whole-genome sequencing will further advance our understanding of the genetic architecture of calcium homeostasis in humans. These findings will likely provide new insight into the complex mechanisms involved in calcium homeostasis and hopefully lead to novel preventive and therapeutic approaches.

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CONFLICT OF INTEREST STATEMENT

This review has not been submitted nor published elsewhere.

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