**UNIVERSITY OF LAUSANNE**

MASTER'S DEGREE IN MEDICINE

# FOCUS ON THE ROLE OF THE CALCIUM-SENSING RECEPTOR (*CASR*) GENE IN BLOOD PRESSURE CONTROL IN HUMANS

STEPHANIE BORNET, 2009-2010

Stephanie.Bornet@unil.ch

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**Tutor**

Bochud Murielle (Murielle.Bochud@chuv.ch)

**Expert**

Bonny Olivier (Olivier.Bonny@chuv.ch)

UNIL | Université de Lausanne
Faculté de biologie et de médecine

CHUV

IUMSP
Institut universitaire de médecine sociale et préventive

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| | |
|---|---|
| 20-HETE | 20-hydroxyeicosatetraenoic acid |
| ACTH | adenocorticotropic hormone |
| ADHH | autosomal dominant hypocalcaemia with hypercalciuria |
| AME | apparent mineralocorticoid excess |
| ANOVA | analysis of variance |
| BMI | body mass index |
| cAMP | cyclic adenosine monophosphate |
| CASR | calcium-sensing receptor |
| CLCKB | chloride channel Kb |
| COX-2 | cyclo-oxygenase 2 |
| DBP | diastolic blood pressure |
| EnaC | epithelial sodium channel |
| FBHH | familial benign hypocalciuric hypercalcemia |
| FIHP | familial isolated hyperparathyroidism |
| GWAS | genome-wide association study |
| LD | linkage disequilibrium |
| MAF | minor allele frenquency |
| MR | mineralocorticoid receptor |
| NCCT | sodium chloride cotransporter |
| NHE3 | sodium proton cotransporter |
| NKCC2 | cotransporter $Na^+K^+2Cl^-$ |
| NSHPT | neonatal severe hyperparathyroidism |
| PKC | protein kinase C |
| PLA2 | phospholipase A2 |
| RAAS | renin-angiotensin-aldosterone system |
| ROMK | renal outer-medullary potassium channel |
| SBP | systolic blood pressure |
| SNP | single nucleotide polymorphism |
| TAL | thick ascending limb of the loop of Henle |
| TRPV4 | transient receptor potential cation channel subfamily V member 4 |
| TRPV5 | transient receptor potential cation channel subfamily V member 5 |

**ABSTRACT**

**Purpose:** The purpose of this work is to analyze the association of variants in the *CASR* gene with blood pressure in CoLaus, a Swiss population-based study. Hypertension is a common disease affecting 20 to 25% of the general population worldwide and represents a major modifiable cardiovascular risk factor.

**Methods:** First, a literature review on hypertension genetics and CASR was conducted using PubMed and Web of Science. Second, a genetic association study of *CASR* variants with blood pressure was conducted. CoLaus is a cross-sectional population-based study aiming at investigating the epidemiology and determinants of cardiovascular risk factors and the metabolic syndrome in a group of people living in Lausanne, Switzerland. The 6,188 subjects included in the study met the following criteria: 1) age between 35 and 75, 2) Caucasian origin and 3) written informed consent. Blood pressure was measured thrice on the left arm after at least ten minutes rest in the sitting position; the average of the two last measurements was used for the analysis. Nuclear DNA was extracted from blood and genotyping was performed using the Affymetrix 500 K SNP chip. Four SNPs located within the *CASR* gene were used: rs11716910, rs10934581, rs5008830 and rs2001548. Data were analyzed using STATA 10. The chi-square test was used to search for an association between each SNP and hypertension. The ANOVA test was used to compare blood pressure in the three genotypes (homozygous for the minor allele, heterozygous and homozygous for the major allele).

**Results:** Blood pressure and hypertension aggregate in families. Several rare monogenic forms of hyper- and hypotension have been described, most of which are associated with disturbed renal sodium handling by the kidney. The *CASR* gene is involved in Bartter's syndrome, a rare monogenic form of hypotension. Yet, more than 90% of hypertension cases are essential and, so far, it has been difficult to identify genetic determinants of essential hypertension. The results of studies using a candidate gene approach have been difficult to reproduce and recently genome-wide approaches have identified new blood pressure candidate genes in humans. In CoLaus, no association between selected *CASR* SNPs and hypertension or blood pressure was found. This might be due 1) to the fact that the CoLaus study is underpowered to detect single SNPs effect for blood pressure, 2) to the fact that only a subset of *CASR* SNPs was used, thus leading to low gene coverage, 3) to the existence of gene-gene or gene-environment interaction (which were not assessed in this project), and/or 4) to the fact that multiple rare unmeasured variants might play a role.

**Conclusion:** Despite negative results in this study, *CASR* remains a potential gene in the understanding of hypertension and blood pressure control in humans. Further association studies in large samples, which would account for all possible *CASR* variants and also consider potential interaction with other genes and with environmental factors are worth considering.

**Keywords:** CASR, hypertension, systolic blood pressure, diastolic blood pressure.

## I) Review of the literature

### 1. Introduction

This work focuses on the role of the calcium-sensing receptor (*CASR*) gene in hypertension in humans. The first part includes a general introduction on hypertension with a description of monogenic forms of hyper- and hypotension, a description of the CASR, its roles and its implications in familial diseases. The second part contains an analysis of data from the population-based CoLaus study. The goal of this analysis is to determine if there is an association between a few single nucleotide polymorphisms (SNPs) in the *CASR* gene and hypertension in this cohort representative of the people living in Lausanne. The association between the continuous blood pressure levels and *CASR* variants is also analyzed.

Monogenic forms of hypertension have been discovered, but most cases of hypertension remain essential. However, we know that this disease has a genetic component, because hypertension aggregates in families. Researchers worldwide are interested in the genetics of hypertension for several reasons, including the facts that hypertension affects 20 to 25% of the population, that it is a modifiable cardiovascular risk factor, that its consequences may be life-threatening and that in most cases, its etiology remains unknown. One long-term perspective would be to imagine a strategy of prevention and treatment based on the genetic background (i.e. a personalized prevention and therapy), but such a strategy is not possible at the time being and it is not clear whether or not this will ever be possible. I chose this topic because of the high prevalence of hypertension in family practice, which is the post-graduate formation I currently intend to follow.

### 2. Methodology

The first step of my master work was to conduct a literature review on calcium homeostasis, the calcium-sensing receptor (CASR) and its relationship with hypertension and/or blood pressure. First, I used articles from the medical website UpToDate[1] as well as articles received from my tutor. Second, I search for articles using the PubMed database. My keywords were "CASR" and "hypertension", linked with the bolean operator "AND". This search retrieved 17 articles. Replacing "hypertension" with "blood pressure" did not identify any further article. Third, I used the database Web of Science to make exactly the same research, but no additional article was retrieved. The number of articles is small because the role of CASR in hypertension has rarely been studied.

As the subject of this work is the genetics of hypertension, I also looked for reviews in PubMed about monogenic forms of hyper- and hypotension. My keywords were "monogenic hypertension" and

[1] www.uptodate.com See complete reference in annex 2

"monogenic hypotension", linked by "AND". I obtained 11 publications, but I worked mainly with a review written by Rosskopf and al. (1) (see chapter 4).
The goal of the following review of the literature is to understand why researchers working on hypertension are interested in the CASR.

## 3. Hypertension[2]

### 3.1. Epidemiology

Hypertension is a highly prevalent disease; 20 to 25 % of the population is affected worldwide. In Switzerland, the Swiss health survey in 2007 showed that 23% of men and 21% of women were once told that their blood pressure was too high (2). In CoLaus, the prevalence reaches 36.7% (3). This is mainly due to the fact that young people under 35 years old are not included in this study and that hypertension prevalence is increasing with age. Men are more likely than women to suffer from this disease. Blood pressure is known to increase with age, especially the systolic blood pressure (SBP); SBP is the most important predictive risk factor for cardiovascular complications. Blood pressure has a circadian rhythm; it usually decreases at night (dipping) and is higher during the early morning (morning surge). Blood pressure may be higher because of physical exercise, psychological stress or emotion. Several mechanisms play a role in the regulation of blood pressure, such as reabsorption of sodium by the kidney, the rennin-angiotensin-aldosterone system or the sympathetic nervous system. Those mechanisms are the target for the currently available anti-hypertensive treatments.

### 3.2. Definition

**Table 1. Classification of hypertension by the European Societies of Hypertension and Cardiology**

| Definition | SBP [mmHg] | DBP [mmHg] |
|---|---|---|
| **Optimal blood pressure** | < 120 | < 80 |
| **Normal blood pressure** | < 130 | < 85 |
| **High normal blood pressure** | < 140 | < 90 |
| **Hypertension grade 1** | 140-159 | 90-99 |
| **Hypertension grade 2** | 160-179 | 100-109 |
| **Hypertension grade 3** | ≥ 180 | ≥ 110 |
| **Isolated systolic hypertension** | ≥ 140 | < 90 |

[2] www.uptodate.com See complete reference in annex 2

People are hypertensive when one of these conditions is fulfilled: 1) SBP ≥ 140 mmHg, 2) diastolic blood pressure (DBP) ≥80 mmHg or 3) taking a medication. The European Societies of Hypertension and Cardiology published guidelines in 2007 classifying hypertension as described in **table 1**.

### 3.3. Hypertension as a cardiovascular risk factor

Hypertension is a modifiable cardiovascular risk factor, as are smoking, diabetes mellitus and dyslipidemia. Non-modifiable cardiovascular risk factors include familial history of cardiovascular disease, sex, age, ethnic group and genetic determinants.

Hypertension is associated with an increased risk (eight to four times) (2) of stroke, cardiac failure, left ventricular hypertrophy, coronary heart disease, chronic kidney disease, abdominal aortic aneurism, arteriopathy of inferior members and hypertensive retinopathy. It is to prevent these complications that hypertension should be detected and treated. The goal of the treatment is to reach a blood pressure level lower than 140/90 mmHg for non-diabetic individuals and lower than 1300/80 mmHg for diabetic individuals, as recommended by the guidelines of PMU (Policlinique Médicale Universitaire) base on three articles[3]. Yet, only half of the affected people are treated and among them, 50% have an uncontrolled hypertension (3) (because of inappropriate treatment or lack of compliance). Hypertension therefore represents a major public health burden worldwide.

### 3.4. Etiology

In 85 to 90% of all cases, hypertension is said to be “essential” or “idiopathic”, because no etiology can be found. Causes of secondary hypertension include hyperaldosteronism, nephropathy, renal artery stenosis, hypercorticism, hyperthyroidism, pheochromocytoma, primary hyperparathyroidism, drug-induced hypertension, pregnancy-induced hypertension and aortic coarctation (40).
Hypertension is a multifactorial disease; environmental factors are implicated in the development of hypertension, but available evidence suggests that this disease has a genetic component. First of all, there is a positive correlation between the blood pressure of parents and children; secondly, blood pressure is more strongly correlated within monozygotic twins than within dizygotic twins; finally,

[3] Chiolero A, Bovet P et Burnier M. Recommandations américaines et européennes pour la prise en charge de patients hypertendus : quel impact pour la pratique ? Revue médicale de la suisse romande, mars 2002. No 551. Disponible sous : titan.medhyg.ch
Chobanian V, Bakris G, Black H et al. The seventh report of the joint national committee on prevention, detection, evaluation and tratment of high blood pressure. JAMA 2003;289:2534-73. Disponible sous: jama.ama-assn.org
Groupe de travail pour la prise en charge de l’hypertension de la société europenne d’hypertension (ESH) et de la société européenne de cardiologie (ESC). ESH/ESC 2007. Recommendations pratiques pour la prise en charge de l’hypertension artérielle. HTA-info, 24, juin 2008 :19-29.

blood pressure is more strongly correlated within biological siblings than within adoptive siblings. However, even if monogenetic forms of hypertension exist (see next paragraph), such forms are rare and most people present a polygenic (i.e. multiple genes each with a small effect) form of hypertension.

## 4. Monogenic forms of hypertension and hypotension

This chapter is mainly inspired by a review of the literature written by Rosskopf and al. (1).

### 4.1. Monogenic forms of hypertension

Only syndromes in which hypertension is the main manifestation are described here (**table 2**) even if hypertension is common in several genetic syndromes.

**Table 2. Monogenic forms of hypertension**

| Disease | Transmission | Gene implicated | Clinic |
|---|---|---|---|
| **Glucocorticoid-remediable hypertension** | Autosomal dominant | Crossing-over between *CYP11B2* and *CYPT11B1* | Volume expansion, metabolic alkalosis, hypokalemia, low plasma renin activity |
| **Apparent mineralocorticoid excess** | Autosomal recessive | Loss-of-function mutations in the gene for HSD11β2 | Hypertension, low renin activity, low aldosterone concentration, metabolic alkalosis |
| **Mineralocorticoid receptor mutations and hypertension exacerbated by pregnancy** | Autosomal dominant | Activating mutation in the mineralocorticoid receptor (MR) | Hypertension during pregnancy |
| **Liddle's syndrome** | Autosomal dominant | *SCNN1B, SCNN1G* (coding for ENaC) | Early-onset hypertension, metabolic alkalosis, hypokalemia, low plasma renin concentration, low aldosterone level concentration |
| **Gordon's syndrome (pseudohypoaldostero-nism type II)** | Autosomal dominant | Gain-of-function mutations in *WNK1;* loss-of-function mutations in *WNK4* | Hypertension, hyperkalemia, hyperchloremic metabolic acidosis, normal glomerular filtration rate, low renin activity |
| **Autosomal dominant hypertension with brachydactyly** | Autosomal dominant | intrachromosomal rearrangement on chromosome 12p. | Hypertension, strokes in young age, brachydactyly, short stature |

*4.1.1. Glucocorticoid-remediable hypertension (GRA) (1,4)*

Glucocorticoid-remediable hypertension (GRA) is an autosomal dominant syndrome due to a crossing-over between two genes with a high degree of homology, *CYP11B2* (coding for aldosterone synthase) and *CYP11B1* (coding for the 11β-hydroxylase). The result is a chimeric gene, consisting of the promoter and regulatory region of the *CYP11B1* followed by the region of *CYP11B2* coding for the structure of aldosterone synthase. As a result, aldosterone is synthesized under the control of ACTH and not, as is the case in a normal physiological situation, under the control of angiotensin II. The treatment consists in glucocorticoids that inhibit ACTH release.

*4.1.2. Apparent mineralocorticoid excess (AME) (1,4,6)*

This autosomal recessive syndrome results from loss-of-function mutations in the gene coding for the hydroxysteroid-11β-dehydrogenase2 (HSD11β2), an enzyme present in tissues sensitive to aldosterone that reduces cortisol to cortisone (an inactive metabolite); this reduction avoids cortisol binding to mineralocorticoid receptors (MR). Both cortisol and aldosterone similarly activate the MR. In AME, cortisol is not appropriately degraded and permanently activates MR in the kidney because of its unusually elevated concentration. This leads to increased sodium reabsorption in the distal nephron.

*4.1.3. Mineralocorticoid receptor mutations and hypertension exacerbated by pregnancy*

In this autosomal dominant disease, there is an activating mutation S810L in the MR (*NR3C2*) itself (1,5,6). This mutation leads to a modification of the affinity of the MR that is now activated by progesterone; as progesterone concentration increases during pregnancy, hypertension is getting the most severe during that period.

*4.1.4. Liddle's syndrome (1,5,6)*

This autosomal dominant syndrome is due to some mutations in the genes *SCNN1B* or *SCNN1G* coding for the carboxy-terminal region of epithelial sodium channel (ENaC, subunit β or γ (5)). This channel is usually present on the apical membrane of the cells in the distal convoluted tubule of the kidney only in presence of aldosterone and is ubiquitinated in absence of aldosterone. These mutations lead to an impaired ubiquitination of ENaC that remains in the membrane even without aldosterone, and sodium and water reabsorption follow, leading to hypertension.

*4.1.5. Gordon's syndrome (pseudohypoaldosteronism type II) (1,4,6)*

This autosomal dominant disease is due to mutations in the *WNK1* and *WNK4* genes, which encode two enzymes implicated in the regulation of the thiazide-sensitive Na-Cl cotransporter (NCCT, gene *SLC12A3*) as well as ROMK (gene *KCNJ1*) and TRPV4 (a calcium channel). WNK4 diminishes the incorporation of the thiazide-sensitive Na-Cl cotransporter in the membrane and WNK1 inhibits this action. A gain-of-function mutation of *WNK1* or a loss-of-function mutation of *WNK4* leads to an

increased membrane expression of the thiazide-sensitive Na-Cl cotransporter, which leads to increased sodium reabsorption and hypertension.

*4.1.6. Autosomal dominant hypertension with brachydactyly (1,4)*

This syndrome has been described in Turkish families presenting severe hypertension and strokes at a young age, as well as brachydactyly and short stature. The locus of this syndrome has been identified on chromosome 12p. It is explained by an impaired ability of the baroreceptor reflex to compensate an increase in blood pressure. Unlike previously described monogenic forms of hypertension, this syndrome is not associated with hypertension via increased sodium and water retention by the kidney as the primary mechanism.

4.2. Monogenic forms of hypotension (1,7-11)

**Table 3. Monogenic forms of hypotension**

| Disease | Transmission | Gene | Defect |
|---|---|---|---|
| Pseudohypoaldostero-nism type I | AR | Inactivating mutations in *SCNN1A, SCNN1B and SCNN1G* | Decrease in ENaC activity. |
| | AD | Loss-of-function mutations in the mineralocorticoid receptor gene | Decrease in MR activity |
| Type I Bartter's syndrome | AR | Inactivating mutation in *SLC12A1* | Decrease in $Na^+K^+2Cl^-$ (NKCC2) activity |
| Type II Bartter's syndrome | AR | Inactivating mutation in *KCNJ1* | Decrease in ROMK activity |
| Type III Bartter's syndrome | AR | Inactivating mutation in *CLCNKB* | Decrease in chloride channel CLCKB activity |
| Type IV Bartter's syndrome | AR | Inactivating mutation in *BSND* | Decrease in chloride channel activity because of mutation of the β-subunit of CLCKB (barttin) |
| Type V Bartter's syndrome | AD | Activating mutation in *CASR* | Abnormal activation of CASR |
| Gitelman's syndrome | AR | Inactivating mutation *SLC12A3* | Decrease in $Na^+Cl^-$ (NCCT) channel |

AD = autosomal dominant; AR = autosomal recessive

As for hypertension, there are monogenic forms of hypotension, summarized in **table 3**. The monogenic forms of hypotension share the common pathophysiological mechanism of being renal

salt-wasting syndromes. Pseudohypoaldosteronism type I (1,4) presents itself with life-threatening neonatal hypotension, dehydration, salt wasting, hyperkalemia, metabolic acidosis and increased aldosterone levels. Gitelman's syndrome is an autosomal recessive disease affecting 1/40,000 people that present with hypokalemia, metabolic alkalosis, hypomagnesaemia and decrease in renal calcium excretion. Bartter's syndrome is a rare genetic diseases affecting 1/1,000,000 people. It is characterized by deficient renal reabsorption of sodium and chloride, hypokalemia, metabolic alkalosis, hypocalcaemia, hypercalciuria and secondary hyperreninaemia and hyperaldosteronism. The pathogenesis of this disease was elucidated in the past ten years and nowadays, five types of Bartter's syndrome are described (see **table 3**). All types are characterized by mutations in genes expressed in the thick ascending limb of the loop of Henle (TAL). Type V Bartter's syndrome is caused by activating mutations in the *CASR.*

In 2002, Vargas-Poussou and al. (10) discovered a *CASR* mutation in a patient suffering from autosomal dominant hypocalcaemia with hypercalciuria (ADHH) and presenting all the characteristics of a Bartter's syndrome. *In vitro* experiments showed that this mutation, L125P, was an activating mutation. Comparing the kinetic of wild-type CASR and mutants CASR, the researchers demonstrated that L125P was the most activating mutation ever described. Then, Watanabe and al. (7) in turn described two further activating mutations of *CASR* in patients with ADHH, A843E and C131W. Those three mutations are associated with more severe clinical features than the ones observed in autosomal dominant hypocalcaemia with hypercalciuria (ADHH) because they permit the activation of the CASR at physiological calcium concentrations, i.e. CASR is much more sensitive to calcium than in patients with ADHH**.**

Ji and al. (8) described mutations in *SLC12A1*, *SLC12A1,* and *KCNJ1* associated with Gitelman's syndrome, type I and type II Bartter's syndromes respectively. In *SLC12A3*, mutations with proven biochemical loss-of-function variants are E112X, R399L, F495L, G613S, G741R, R964Q and G989R; they lead to loss of salt, lower blood pressure and protection against hypertension. R320FS in *SLC12A1* and H251Y and T313FS is *KCNJ1* are variants in which loss-of-function has been demonstrated in vitro (8).

### 4.3. Polygenic forms of hypertension

For about twenty years, researchers have been looking for genes responsible for hypertension. Many variants in genes coding for molecules implicated in the regulation of salt retention have been discovered. The molecules that are possibly implicated are components of the classical blood pressure

regulation pathways like the renin-angiotensin-aldosterone system, renal transporters, receptors of the sympathetic nervous system, but also G-proteins, hormones and enzymes. So far, more than 100 genes have been studied and results have often been inconsistent across studies using a candidate gene approach. A more detailed list of molecules included in polygenic forms of hypertension is available in articles written by Luft (5) , Lang and al.(4) and the review written by Rosskopf and al. (1).

More recently, a hypothesis-free genome-wide approach has been possible thanks to the availability of genome-wide chips containing 500'000 or 1'000'000 SNPs. Genome-wide association studies (GWAS) have already identified single nucleotide polymorphisms (SNPs) robustly associated with blood pressure. Two recent studies have been published in *Nature Genetics* (12,13). In 2009, the Global BPgen consortium (12) conducted a meta-analysis of GWAs on hypertension including 17 cohorts of 23,433 Caucasians, and tested 2.5 million SNPs for association with SBP, DBP and hypertension. Eight loci were identified as being associated with SBP or DBP: *CYP17A1, CYP1A2, FGF5, SH2B3, MTHRF, c10orf107, ZNF625 and PLCD3*. The CHARGE consortium (13) conducted a meta-analysis including 29,136 participants from six prospective cohorts and identified 13 SNPs associated with SBP, 20 SNPs associated with DBP and 10 SNPs associated with hypertension. Four SNPs were associated with SBP in both studies (*STP2B1, CYP17A1, PLEKHA7* and *SH2B3*) and six with DBP in both studies (*ATP2B1, CACNB2, CSK-ULK3, SH2B3, TBX3-TBX5* and *ULK4*).

Several conclusions can be drawn from these genome-wide association studies. The first observation is that the variants in genes known to cause hypertension (i.e. the genes coding for the molecules targeted by current anti-hypertensive therapies) were not significantly associated with blood pressure. The actual treatment of hypertension consists of inhibitors of the renin-angiotensin system, diuretics, inhibitors of the sympathetic nervous system and blockers of calcium channels. Mutations in genes coding for components of those regulatory systems known to control blood pressure did not appear either. The second observation is that a great number of variants must be analyzed to discover only few SNPs associated to hypertension. To take into account the very large number of statistical tests conducted and avoid false positive results, researchers need to use a very low threshold for the p-value for considering an association to be significant. The third observation is that the effect sizes of the variants associated with blood pressure was very small. As a consequence, very large sample sizes (> 20,000) need to be used in order to have enough power to detect such small effect sizes. This also implies that any singly variant only has a small impact at the individual level. The fourth observation is that genome-wide approaches, unlike candidate gene approaches, are hypothesis free and therefore allow identifying genes with previously unknown function and genes previously unsuspected to play a role in blood pressure control. Genome-wide association studies may be a starting point to discover new pathways and mechanisms for controlling blood pressure level, but this is usually not the case at the time being. The fifth observation is that GWAs did not consider the interaction between genes and

the ones between genes and environment so far. SNPs are analyzed separately from each other and only the association between one particular genetic variant and blood pressure is considered. It is therefore likely that a lot of information is being lost.

## 5. The calcium-sensing receptor (CASR)

### 5.1. Structure (14,15)

The calcium-sensing receptor is a G-protein-coupled receptor of 1,078 amino acids (see **figure 1**). It was first isolated from bovine parathyroid in 1993 (16,17). There is high homology between the bovine and the human forms of the CASR. The receptor is composed of three parts. The extracellular domain, made of about 610 amino acids, contains clusters of amino-acids interacting with calcium. The N-glycosylation of these clusters can modify the level of activation of the receptor. Type I calcimimetics (see § 5.3.) bind to this extracellular domain. The second part of the receptor contains the 7 transmembrane domains and several intracellular loops involved in the receptor phosphorylation. This second part of the receptor contains the site of binding of the calcimimetics type II. The C-terminal intracellular part of the receptor is composed of 200 amino-acids. Some sequences in the internal domain serve for the phosphorylation of the CASR by PKC, that diminishes the level of activation of this receptor.

### 5.2. Localization and roles of the CASR (9,14,18)

The most studied role of the CASR is its function in the parathyroid cells, where it senses extracellular ionized calcium concentration to modulate the secretion of the parathyroid hormone (PTH); it increases PTH secretion when calcaemia decreases and vice versa. PTH maintains calcium level in a narrow range by three mechanisms: 1) it enhances bon resorption, 2) it enhances calcium reabsorption by the kidney and 3) it promotes the 1α-hydroxylation of 25-OH-vitamin D in the kidney, leading to (1,25)-$(OH)_2$-vitamin D, the active form of this molecule; vitamin D then enhances the reabsorption of calcium in the gut and by the kidney. The CASR signaling in the parathyroid cells is mediated by G-proteins, especially $G_{q/11}$ (mainly) and $G_i$. The result is the activation of the phospholipase C (PLC) that stimulates the formation of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 induces the exit of calcium from the endoplasmic reticulum, resulting in an elevation of calcium concentration in the cytosol, and DAG activates PKC. (see **figure 2**).

In the parathyroid glands, CASR also prevents gland hyperplasia; that was proven by the fact that hetero- and homozygous carriers of *CASR* inactivating mutations may have parathyroid hyperplasia

(14). The CASR is also implicated in the secretion of calcitonine (19) when the serum calcium concentration is high.

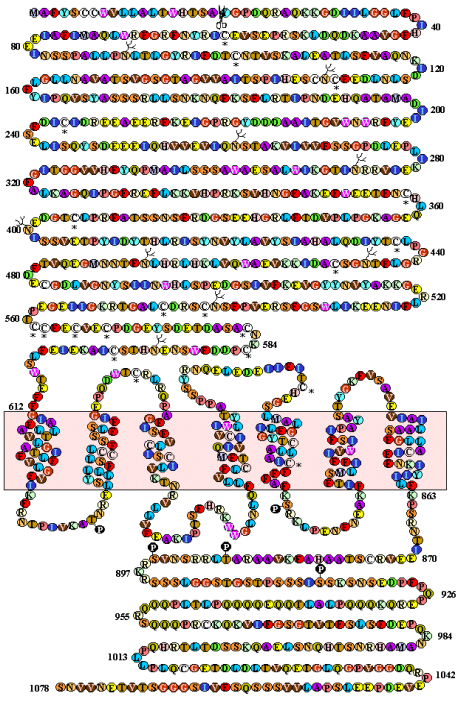

**Figure 1. GPCR topology of the CASR[4]**

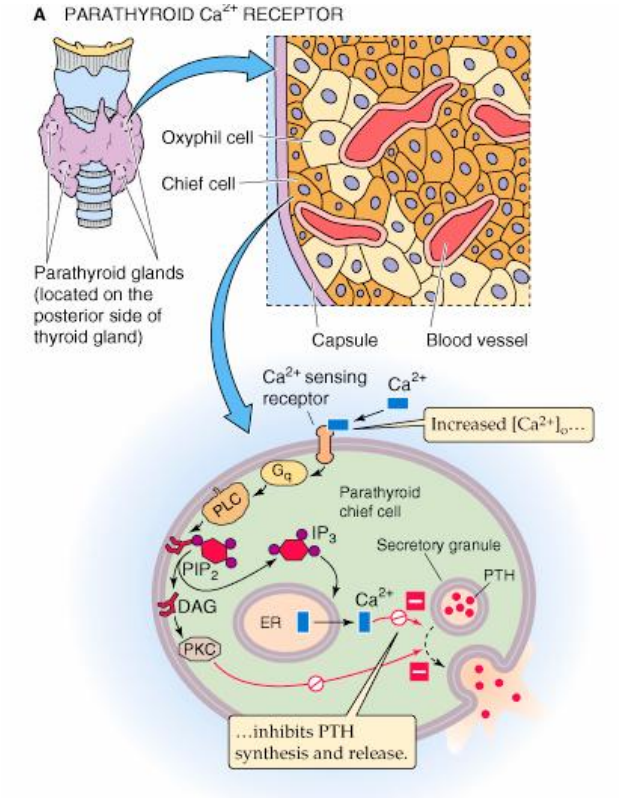


**Figure 2. CASR signaling in the parathyroid gland[5]**

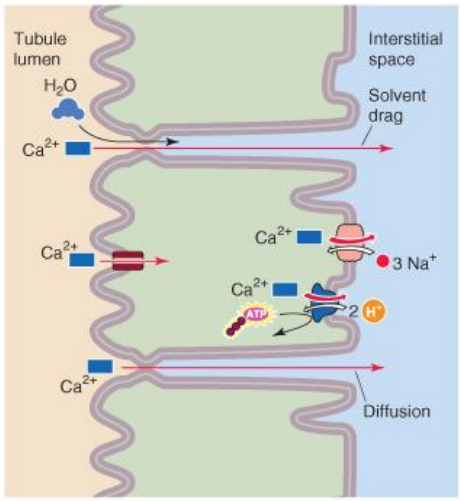


**Figure 3. Reabsorption of calcium in the proximal tubule[6].**

[4] See reference in annex 1
[5] See reference in annex 1
[6] See reference in annex 1

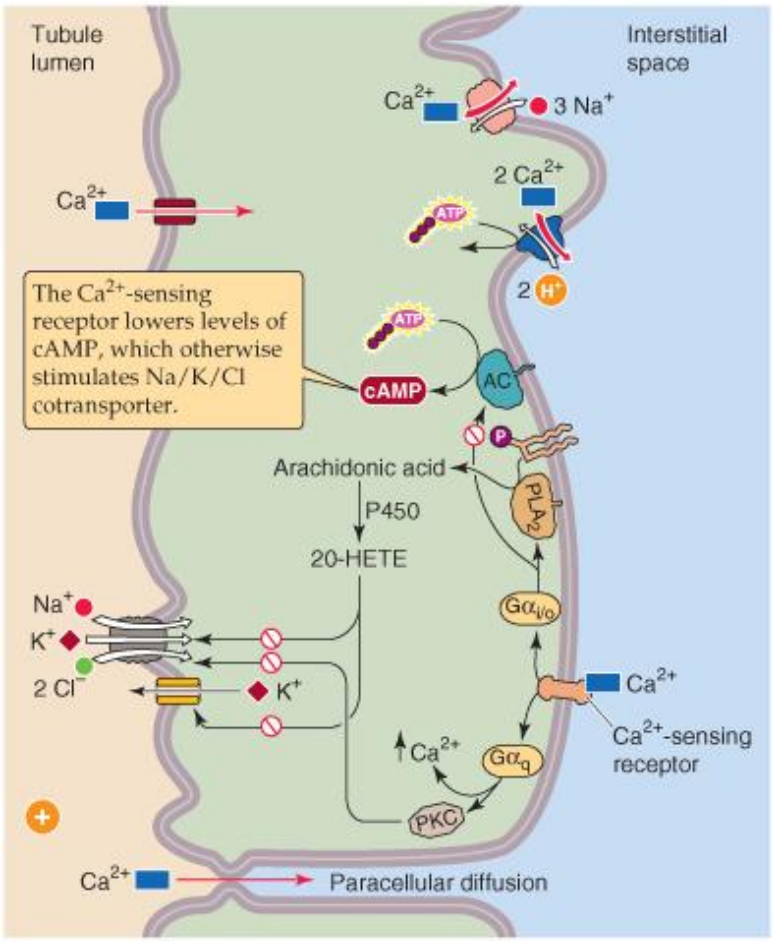


**Figure 4. Reabsorption of calcium in the TAL**[7]

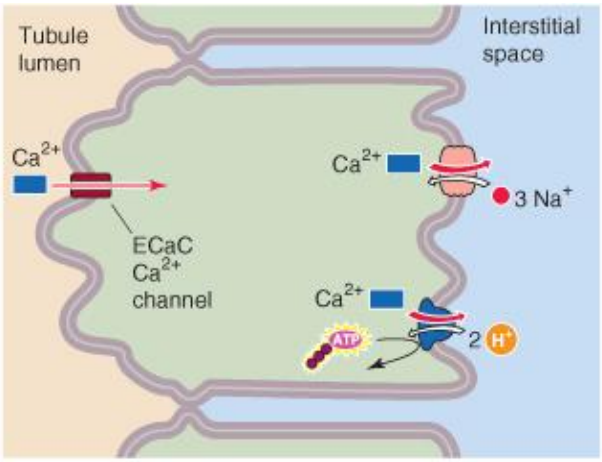


**Figure 5. Reabsorption of calcium in the DCT**[8]

The CASR is also localized in the nephron, at the apical membrane of the proximal tubule, the cortical and inner medullary collecting ducts and at the basolateral membrane of the thick ascending limb of the loop of Henle (TAL). The signaling pathway is also mediated by G-proteins. When the CASR is coupled with Gαi, cAMP formation decreases and the consequence is the inhibition of the $Na^+K^+2Cl^-$ cotransporter (NKCC2) and the renal outer medullary potassium channel (ROMK). When it binds to

[7] See reference in annex 1
[8] See reference in annex 1

$G_{\alpha q}$, PKC is activated and inhibits NKCC2. When CASR is bound to $G_i/G_o$, the consequence is the activation of the phospholipase type 2. PLA2 increases the degradation of arachidonic acid in 20-hydroxyeicosatetraenoic acid (20-HETE) by the P450 or in prostaglandins by the COX-2. The role of 20-HETE is to inhibit NKCC2. However, under normal conditions, 20-HETE formation is favored, but when CASR is activated, the COX-2 pathway is predominant (it means that calcium is reabsorbed). In conclusion, the pathways through $G_{\alpha i}$ and $G_{\alpha q}$ work together to excrete calcium as the $G_i/G_o$ pathway leads to reabsorption of calcium.

In the proximal tubule (see **figure 3**), the CASR is expressed in the apical membrane, where it is implicated in the reabsorption of phosphate, by antagonizing the effect of the PTH (18). In this part of the nephron, sodium is reabsorbed via the $Na^+/H^+$ cotransporter (NHE3). This transporter is inhibited by the PTH. The current hypothesis is that CASR may enhance sodium reabsorption by inhibiting the PTH effect (as it inhibits the PTH effect on the phosphate reabsorption). In the TAL (see **figure 4**), the CASR is localized on the basolateral membrane, where its activation inhibits NKKC2, ROMK and $Na^+K^+$ATPase. The consequence is a diminution of the gradient for calcium reabsorption by a less positive lumen and a reduction of urine osmolarity. In the distal convoluted tubule (see **figure 5**), the CASR is expressed on the basolateral membrane, but its role in this part of the kidney is not well-defined. There, calcium is reabsorbed by the TRPV5 channel and sodium via the thiazide-sensitive channel NCC (a cotransporter $Na^+/Cl^-$). Both channels are regulated by WNK kinases. PTH activates the TRPV5, and calcimimetics compounds reduce TRPV5 activation level in experiments, but the mechanism is not fully understood. Note that when CASR is activated in both TAL and distal convoluted tubules, it inhibits the action of PTH (that reabsorbs calcium) in hormone-sensitive sites of the nephron. The CASR is also expressed in the collecting ducts, but its function is not defined. In the macula densa, the CASR expressed on the basolateral membrane may act by sensing the interstitial composition, affecting renin release and the tone of afferent arterioles. Maillard and Burnier (29) recently suggested that CASR activation may decrease renin release, i.e. that CASR may be implicated in the regulation of blood pressure via the renin-angiotensin-aldosterone system.

The role of the CASR in the regulation of sodium balance is still not fully elucidated. Desfleurs's hypothesis (21) is that CASR plays a role in sodium reabsorption indirectly: as it inhibits NKCC2 in the TAL, calcium and magnesium are not reabsorbed in this part of the nephron; their delivery at the distal part of the nephron then interferes with sodium reabsorption. However, other researchers seem to say that CASR interferes directly with sodium reabsorption in the TAL. The *in vivo* observation suggests that hypercalcaemia results in both increased calciuria and sodium excretion.

In the intestine (14,18,19), CASR modifies the transport of solutes and modulates the proliferation and differentiation of the epithelial cells of the colon. In the placenta (14,22) it maintains calcium homeostasis. It is also implicated in the transport of calcium in breast epithelial cells. Finally, CASR plays a role in cell proliferation, cell differentiation and apoptosis in several other tissues (14,15).

5.3. Agonists and antagonists of CASR (14,15,23)

Not only calcium, but also other ligands can bind to CASR (see **table 4**). Those ligands are classified in calcimimetics or calcilytic agents. Type I calcimimetics are full agonists that bind to the extracellular domains of CASR; there are divalent cations, trivalent cations, polycations and polybasic amino acids. Type II calcimimetics are allosteric modulators; while binding to CASR, they induce a conformational change in the tridimensional structure of the receptor and lower its threshold of activation. Those molecules are used to treat hyperparathyroidism because they inhibit PTH secretion. Calcilytic agents are allosteric inhibitors of CASR used to increase the pulsatil secretion of PTH. Balan and al. (24) have recently tried to synthesize negative allosteric modulators of the CASR that could be used in the treatment of osteoporosis. PTH increases bone turnover (it increases formation of bones more that resorption). The advantage of this treatment is not its mechanism of action, but relies on the fact that the actual treatment of osteoporosis with biphosphonates is made of injections while calcilytic agents can be taken orally.

**Table 4. Agonists and antagonists of the calcium-sensing receptor**

| | **Role** | **What** | **Examples** | **Effect** |
|---|---|---|---|---|
| **Type I calcimimetics** | Full agonists | Divalent cations | $Ca^{2+,}$ $Mg^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Fe^{2+}$, $Ni^{2+}$, $Pb^{2+}$ | ↓ PTH |
| | | Trivalent cations | $Gd^{3+}$, $La^{3+}$, $Eu^{3+}$, $Tb^{3+}$, $Yt^{3+}$ | ↓ PTH |
| | | Polycations | spermine, aminoglycosides | ↓ PTH |
| | | Polybasic aminoacids | | ↓ PTH |
| **Type II calcimimetics** | Allosteric modulators | | Cinacalcet | ↓ PTH |
| **Calcilytic agents** | Allosteric inhibitors | | Ronacaleret, NPS 2143, JTT305 | ↑ |

5.4. *CASR* gene

The human *CASR* is localized on chromosome 3q13.3-21. On the CASR mutation database[9], 112 mutations have been described, including 98 missense, 6 nonsense, 8 insertions or deletion and one splice mutation. Those mutations play a role in healthy individuals as well as in people affected by a familial disease.

[9] http://www.casrdb.mcgill.ca/

First of all, *CASR* polymorphisms are implicated in serum calcium in healthy individuals. In 1998, Cole and al. (25) demonstrated that three polymorphisms located in exon 7 (A986S, R990G and Q1011E) were in linkage disequilibrium. They studied the role of the polymorphism A986S in calcium concentration in healthy women and showed that it was associated with calcium corrected for albumin: carriers of the S allele (i.e. genotypes AS and SS) have a higher calcium concentration than people homozygous for the wild-type allele, even after adjustment for the pH. They concluded that the A986S polymorphism is one of the determinants of plasma calcium concentration and that the S-allele is associated with higher calcium level and a decreased calcium excretion by the kidney. In this same exon, Cole (25) identified the insertion of an *Alu* sequence in exon 7 and demonstrated the loss of intracellular signaling in presence of those repetitive elements. In 2006, Kelly (26) assessed the prevalence of those polymorphisms in the West of Scotland and demonstrated that there were similar to those previously described for Caucasian population; he also confirmed the results of Cole and al. about the role of *CASR* in serum calcium in healthy individual.

Mutations in *CASR* have been shown to cause familial diseases (11,15,27) (see **table 5**). A loss-of-function *CASR* mutation implies that a higher calcium concentration is necessary to inhibit PTH secretion and to excrete calcium through the kidney (the receptor is less sensitive to calcium). Several diseases have this characteristic. Familial benign hypocalciuric hypercalcemia (FBHH) is an autosomal dominant disease due to inactivating mutations in the *CASR* gene (11) Over forty mutations (27) have already been discovered, half of them affecting the extracellular domain. Each family has its own mutation, even if three polymorphisms (P55L, T138M and R185Q) have been discovered in unrelated families (27). Affected patients present mild hypercalcemia, hypocalciuria, inappropriately normal PTH level and elevated magnesium level[10].

Neonatal severe hyperparathyroidism (NSHPT) is an autosomal recessive disorder present in carriers of homozygous inactivating mutations in the *CASR* gene. Infants present with high PTH level at birth (more than ten times above normal), severe hypercalcemia and hypocalciuria; complications include bone demineralization. The disease is more severe than FBHH and can be fatal without a rapid parathyroidectomy[11].

The last disease due to loss-of-function mutations in the *CASR* gene is familial isolated hyperparathyroidism (FIHP); five mutations (T1001L, K336- deletion, L650P, V689M and F881L (11)) have been described.

[10] "Disorders of the calcium-sensing receptor: Familial hypocalciuric hypercalcemia and autosomal dominant hypercalcemia" on UpToDate
[11] "Disorders of the calcium-sensing receptor: Familial hypocalciuric hypercalcemia and autosomal dominant hypercalcemia" on UpToDate

On the other hand, activating mutations of the *CASR* gene lead to autosomal dominant hypocalcaemia with hypercalciuria (ADHH) or type V Bartter's syndrome. ADHH is a disorder with low PTH level, hypocalcaemia, hypercalciuria, recurrent nephrolithiasis, and sometimes low magnesium concentration. Over twenty-five mutations (27) are described. As in FBHH, each family has its own mutation, but shared mutations exist (T151M, N118K, F612S and E127A (27), that is the first mutation described as causing ADHH). As described in paragraph 4.2., type V Bartter's syndrome clinically includes hypokalemic alkalosis, renal salt wasting, hypotension, hyperaldosteronism, high renin concentration, increased prostaglandin excretion, hypercalciuria and nephrocalcinosis. This type of Bartter's syndrome is due to activating mutations in *CASR.*

**Table 5. Mutations in the *CASR* gene causing calcium disorders**

| Disease | Type of mutation | Polymorphisms (examples) | Clinic |
|---|---|---|---|
| **FBHH** | Loss-of-function | Heterozygous mutations P55L, T138M, R185Q | Hypercalcemia, hypocalciuria, normal PTH, elevated magnesium |
| **NSHPT** | Loss-of-function | Homozygous mutations P55L, T138M, R185Q | Severe hypercalcemia, severe hypocalciuria. |
| **FIHP** | Loss-of-function | T110L, K336- deletion, L650P, V689M and F881L | Hypercalcaemia, hypercalciuria, inappropriately high PTH levels, and isolated parathyroid tumors with no evidence of hyperfunction of any other endocrine tissues |
| **ADHH** | Gain-of-function | T151M, N118K, F612S, E127A | Low PTH level, hypocalcaemia, hypercalciuria, recurrent nephrolithiasis, and sometimes low magnesium concentration |
| **Bartter's syndrome type V** | Gain-of-function | A843E (15) | Hypokalemic alkalosis, renal salt wasting, hypotension, hyperaldosteronism, high renin concentration, increased prostaglandin excretion, hypercalciuria and nephrocalcinosis |

Note that auto-immune activation of the CASR is responsible for autoimmune hypoparathyroidism.

At the time being, it is not clear to what extend *CASR* variants play a role in bone mineral density(28), coronary artery disease (29), controlling serum calcium (especially R990G and Q1011E) (30,31) and PTH levels in primary hyperparathyroidism (espectiallyR990G and Q1011E) (31,32).

# II) CoLaus data analysis

## 1. Methodology

### 1.1. CoLaus : methodology (3)

The CoLaus study is a cross-sectional population-based study whose aim is to investigate the epidemiology and the genetic determinants of cardiovascular risk factors and of the metabolic syndrome.

The recruitment took place in Lausanne, a city of 117,161 inhabitants. It lasted from June 2003 to May 2006. A first selection was made: only people between 35 and 75 years old were to be potentially included in the study. The CoLaus study is not only interested in the prevalence of hypertension, but also in cardiovascular risk factors in general. Preanalytical calculations showed that about 6,000 people had to be included in the study to detect genetic associations with diseases with a prevalence of about 15% (as the prevalence of hypertension is 20 to 25%, there was a good chance to discover an association).

Among the source population (i.e. every person living in Lausanne between 35 and 75 years), 19,830 (35%) received a letter inviting them to participate to the study. Among them, 54 were considered as ineligible (because they were not living in Lausanne anymore, because they were dead or did not meet the age criteria); 4,667 were considered as non responders (i.e. that no contact was established after two letters and three telephone calls). Among the remaining 15,109 people, 6,189 refused to participate (41% of responders) and 799 were ineligible (because of moving, age criteria or death). At this stage, 8,121 agreed to participate, which represents 41% of the persons who received an invitation. Finally, among persons who accepted to participate, 1,383 were not included because the goal was to have about 6'000 participants and because 549 were not Caucasians. The final cohort includes 6,188 subjects.

Three inclusion criteria were necessary to participate in the study: 1) age between 35 and 75 years, 2) Caucasian origin (i.e. having both parents and grandparents born in a restricted list of countries) and 3) written informed consent.

Among the data obtained in this study, the blood pressure level is the most important for my work. The blood pressure was taken three times on the left arm after a ten-minute rest in a sitting position

with an Omron® HEM-907 automated oscillometric sphygmomanometer (Matsusaka, Japan). This sphygmomanometer was validated for blood pressure measuring in 2002 by El Assaad and al. (35). Hypertension was defined in accordance to The European Societies of Hypertension and Cardiology published guidelines described previously. DNA was extracted from leukocytes obtained by a venous blood sample of 50 milliliters and genotyping was performed using Affymetrix 500 K SNP chip. Four SNPs located within and around the *CASR* gene were selected because of their strong association with serum calcium in CoLaus.

### 1.2. Statistical analysis

For this study, only a few variables were kept out of the entire database: age, sex, hypertension status, serum calcium, corrected serum calcium, proteins, albumin, serum creatinine, systolic and diastolic blood pressures, treatment status for angiotensin-converting enzyme inhibitors, beta-blockers, diuretics and/or calcium-channel blockers. Four *CASR* SNPs were selected: rs11716910, rs10934581, rs5008830 and rs2001548. Those SNPs were selected because they have the strongest association with corrected serum calcium in CoLaus. The p-value for the $X^2$ test less than

The analysis was made using Stata version 10 (Statacorp, College Station, TX, USA). First of all, the population was described, the proportion of women and men compared, the mean age, the proportion of hypertensive among this population and among hypertensive and the percentage of hypertensive under treatment calculated. Then, hypertensive and normotensive were compared for age, weight, height, BMI, systolic and diastolic blood pressure, and protein, albumin, total calcium, corrected serum calcium and creatinine concentrations.

Then, numeric variable were generated with the SNPs, using an additive model: the value 2 was attributed to the less frequent homozygous genotype (i.e. homozygous for the minor allele), the value 1 to the heterozygous phenotype and the value 0 to the homozygous phenotype for the major allele. The next step was to see if genotypes were in Hardy-Weinberg equilibrium by doing a $X^2$ test for goodness of fit between observed and predicted genotypic frequencies. This test is based on the Hardy-Weinberg distribution, that represents the mathematical relationship between allele frequencies and genotype frequencies seen when no distorting factors are present. The criteria necessary to use Hardy-Weinberg proportions are 1) there is no segregation distortion, 2) the allele frequencies are the same in men and in women, 3) there is no mutation, 4) there is no migration 5) there is no selection 6) the population size is big and 7) there is random mating. In this test, the hypothesis (H0) is that the genotypes are in equilibrium in the population. If the p-value if the $X^2$ test is $<0.05$, H0 of Hardy-Weinberg equilibrium is rejected, which means that genotypes are probably not in equilibrium. If the

p-value is >0.05, H0 is accepted, which means that genotypes are probably in the Hardy-Weinberg equilibrium. Only SNPs that are in Hardy-Weinberg equilibrium are usually used for further statistical analyses.

To avoid duplicate analysis, SNPs were tabulated one with the other to see if there was a correlation, i.e. carrying one genotype at one SNP involves always carrying a specific genotype at the second SNP. In case of redundancy, only one of the SNPs can be used for further analyses. When two SNPs are close enough on the same chromosome, they are usually transmitted together during meiosis. When two SNPs do not randomly segregate in the population, they are said to be in linkage disequilibrium (LD). LD is the non random association of two or more loci in a population. $r^2$ is used to quantify the LD. The higher the $r^2$ is, the closer the two SNPs are on the chromosome and in this case, they have great chance to be transmitted together. $r^2$ ranges from 0 (no LD) to 1 ("complete" LD). For this test, I calculated the $r^2$ for: rs11716910 and rs10934581, rs11716910 and rs5008830, rs11716910 and rs2001548, rs10934581 and rs5008830, rs10934581 an rs2001548 and rs5008830 and rs2001548.

To see if SNPs are associated with hypertension, a $X^2$ test was made to compare the repartition of the genotypes between normotensive and hypertensive. The null hypothesis (H0) is that the genotype distributions are similar in both groups. A p-value <0.05 was considered as statistically significant and suggested an association between the SNP and hypertension, because H0 would be rejected. A further analysis consisted in conducting stratified analyses using a $X^2$ test to compare the distribution of genotypes across subgroups: younger versus older than 55 years old, male versus female, BMI $\geq$ 30kg/m$^2$ versus BMI < 30kg/m$^2$ (i.e. obese versus non obese), corrected calcium concentration < 2.21mM versus $\geq$ 2.21mM and creatinine concentration < 78.1mM versus $\geq$ 78.1mM (2.21mM and 78.1mM are the median values for corrected calcium and creatinine concentration, respectively). The population was divided in two subgroups for each value, and not tertiles or quartiles, in order to have groups big enough to be able to detect a relatively small effect of the SNPs on hypertension. These stratified analyses aimed at exploring the presence of effect modification of selected covariates (e.g. sex, obesity) on the association of *CASR* variants with hypertension.

The second question of this analysis was to see if there is a difference in the mean systolic and diastolic blood pressures across the three genotypes (homozygous for the minor allele, homozygous for the major allele and heterozygous). To answer this question, the ANOVA test was used. H0 in this test is that mean values in the different subgroups (here, the three genotypes) are the same. If the p-value of an ANOVA test is <0.05, H0 is rejected, it means that values in the different subgroups are different. As for the $X^2$ test, stratification was then made for the same variables with the same subgroups to explore potential effect modifications

## 2. Results

### 2.1. Description of the study sample

Among the 6,188 participants, 47,5% were men and 52,5% women. The mean age (SD) of participants was 53.1 years old (53.1 ± 10.8). Men were about one year younger than women (mean age of 52.6 and 53.5 years respectively). The mean height of the studied population is the same in hypertensive and normotensive, but the weight differs between both groups, with hypertensive participants being 8.6 kg heavier than normotensive participants (**table 6**). Therefore, the mean BMI in hypertensive participants is 3 $kg/m^2$ higher than in normotensive participants (this is not surprising, given that weight gain may lead to higher blood pressure level). 35.94% of the participants are hypertensive, i.e. that their blood pressure was ≥ 140/90mmHg or they were on treatment at the time of measurement. However, 14.2% of the population is treated for hypertension, which means that only 35.9% of hypertensive participants receive a medication. 52.03% of the population is in overwight (BMI ≥ 25 $kg/m^2$). This table gives the values for each variable in the all sample, in hypertensive and in normotensive. Hypertensive and normotensive were not compared to look for statistical differences.

**Table 6. Description of the study sample**

| Variables | All | Hypertensive | Normotensive |
|---|---|---|---|
| **N** | 6,188 | 2,224 (35.94%) | 2,964 (64.06%) |
| **Age [years]** | 53.1 (10.8) | 60.0 (9.8) | 49.8 (9.9) |
| **Sex** | 47.5% M<br>52.5% F | 55.6% M<br>44.4%F | 43% M<br>57% F |
| **Weight [kg]** | 73.6 (15.1) | 79.1 (15.9) | 70.5 (13.7) |
| **Height [cm]** | 168.6 (9.3) | 168.2 (9.3) | 168.8 (9.3) |
| **BMI [$kg/m^2$]** | 25.8 (4.6) | 27.9 (4.8) | 24.7 (4.0) |
| **Systolic blood pressure [mmHg]** | 128.3 (17.9) | 144.5 (16.5) | 119.2 (10.8) |
| **Diastolic blood pressure [mmHg]** | 79.3 (10.8) | 87.2 (11.0) | 74.9 (7.8) |
| **Hypertension treatment (%)** | 14.20% | 39.52% | 0% |
| **Protein [g/l]** | 74.41 (4.40) | 75.29 (4.48) | 73.92 (4.26) |
| **Albumin [g/l]** | 44.20 (2.53) | 44.25 (2.54) | 44.16 (2.52) |
| **Calcium [mM]** | 2.29 (0.10) | 2.31 (0.10) | 2.27 (0.10) |
| **Corrected calcium [mM]** | 2.21 (0.90) | 2.23 (0.10) | 2.20 (0.90) |
| **Creatinine [µM]** | 80.01 (21.66) | 83.06 (30.57) | (78.30 (14.13) |

Data are mean values with standard deviation in parenthesis, unless specified otherwise.

Given that normal calcium ranges from 2.2 to 2.5mM, albumin from 40 to 60g/l, creatinine from 50 to 110µM and total proteins from 60 to 80g/l, 15,7% of participants have hypocalcemia and 1,4% hypercalcemia (9,8% and 0,4% for corrected calcium respectively), 3,2% have hypoalbuminemia, 178 subjects (2.9%) have a creatinine above 110 µM (among them, 9 have a value above 200) and 470 subjects (7.6%) have an hyperproteinemia; those participants were included in the analyses. Mean values for the studied variables are given in **table 6**.

2.2. Hardy-Weinberg proportions

The results of the $X^2$ test for goodness of fit between observed and predicted genotypes for the four selected SNPs in the *CASR* gene are given in **table 7**. This table also shows the number of people carrying the three possible genotypes (heterozygous, homozygous for the minor allele and homozygous for the major allele).

**Table 7. Hardy-Weinberg equilibrium test**

| SNP | AA | Aa | Aa | Total | MAF | P |
|---|---|---|---|---|---|---|
| **rs11716910** | 2257 | 2446 | 617 | 5320 | 34.59% | 0.2400 |
| **rs10934581** | 2335 | 2438 | 660 | 5433 | 34.58% | 0.5428 |
| **rs5008830** | 3550 | 1629 | 203 | 5382 | 18.91% | 0.3444 |
| **rs2001548** | 3516 | 1589 | 148 | 5253 | 17.64% | 0.0480* |

Data are the number of people carrying the different genotypes. AA = homozygous genotype for the major allele; Aa = heterozygous genotype; aa= homozygous genotype for the minor allele; MAF = minor allele frequency; P=p-value for $X^2$ test; the minor allele is G for rs11716910, T for rs10934581, A for rs2001548, A for rs5008830.

The minor allele frequencies (MAF) are above 15% for the four studied SNPs. It means that a sufficient number of participants carry this allele and that if an allele is associated with hypertension, SBP or DBP, there will be a sufficient number of participants to detect this effect. On the contrary, if the MAF was 1% for example, only 60 participants of CoLaus would carry this allele.

The genotypes of one SNP (rs2001548) do not follow the Hardy-Weinberg proportion. Three main reasons may explain this result. The first one is that one condition underlying the low of Hardy-Weinberg is not respected in the CoLaus population. The second explanation may be a genotyping error; to exclude this hypothesis, genotyping should be repeated, but for technical reasons, this cannot be done for this work. The third reason is a chance finding. The consequence of this result is that this SNP will not be used for further analyses.

2.3. Linkage disequilibrium between SNPs

The further tables (**tables 8 to 13**) give the results of tabulation of the four SNPs two by two. The $r^2$ is calculated here to avoid redundant calculations for further analyses. The conclusion is that there is a strong correlation between rs11716910 and rs10934581 and between rs5008830 and rs2001548 (see **tables 8 and 13**). rs2001548 was previously excluded and rs5008830 will be used for further analyses. Among the remaining two SNPs, rs10934581 is selected for further analysis because of its higher p-value for the Hardy-Weinberg test. In conclusion, further analysis will be made using rs10934581 and rs2008830 only.

**Table 8. Cross-tabulation of rs11716910 and rs10934581**

| **rs10934581**<br>**rs11716910** | **C/C** | **T/C** | **T/T** | **Total** | |
|---|---|---|---|---|---|
| **A/A** | 2254 | 3 | 0 | 2257 | |
| **G/A** | 34 | 2406 | 6 | 2446 | |
| **G/G** | 0 | 15 | 602 | 617 | |
| **Total** | 2288 | 2424 | 608 | 5320 | $r^2 = 0.98$ |

Data are the number of people carrying the genotypes.

**Table 9. Cross-tabulation of rs11716910 and rs5008830**

| **rs5008830**<br>**rs11716910** | **G/G** | **A/G** | **A/A** | **Total** | |
|---|---|---|---|---|---|
| **A/A** | 1245 | 842 | 150 | 2237 | |
| **G/A** | 1690 | 683 | 49 | 2422 | |
| **G/G** | 530 | 76 | 2 | 608 | |
| **Total** | 3465 | 1601 | 201 | 5267 | $r^2 = 0.05$ |

Data are the number of people carrying the genotypes.

**Table 10. Cross-tabulation of rs11716910 and rs2001548**

| **rs2001548**<br>**rs11716910** | **G/G** | **A/G** | **A/A** | **Total** | |
|---|---|---|---|---|---|
| **A/A** | 1231 | 825 | 107 | 2163 | |
| **G/A** | 1669 | 666 | 38 | 2373 | |
| **G/G** | 532 | 70 | 1 | 603 | |
| **Total** | 3432 | 1561 | 146 | 5139 | $r^2 = 0.05$ |

Data are the number of people carrying the genotypes.

**Table 11. Cross-tabulation of rs10934581 and rs5008830**

| rs5008830<br>rs10934581 | G/G | A/G | A/A | Total | |
|---|---|---|---|---|---|
| **C/C** | 1291 | 871 | 152 | 2314 | |
| **T/C** | 1689 | 676 | 48 | 2413 | |
| **T/T** | 569 | 81 | 3 | 653 | |
| **Total** | 3549 | 1628 | 203 | 5380 | $r^2$= 0.05 |

Data are the number of people carrying the genotypes.

**Table 12. Cross-tabulation of rs10934581 and rs2001548**

| rs2001548<br>rs10934581 | G/G | A/G | A/A | Total | |
|---|---|---|---|---|---|
| **C/C** | 1277 | 854 | 109 | 2240 | |
| **T/C** | 1669 | 659 | 38 | 2366 | |
| **T/T** | 569 | 75 | 1 | 645 | |
| **Total** | 3515 | 1588 | 148 | 5251 | $r^2$= 0.05 |

Data are the number of people carrying the genotypes.

**Table 13. Cross-tabulation of rs5008830 and rs2001548**

| rs2001548<br>rs5008830 | G/G | A/G | A/A | Total | |
|---|---|---|---|---|---|
| **G/G** | 3468 | 1 | 0 | 3469 | |
| **A/G** | 2 | 1571 | 0 | 1573 | |
| **A/A** | 0 | 16 | 147 | 163 | |
| **Total** | 3470 | 1588 | 147 | 5205 | $r^2$= 0.99 |

Data are the number of people carrying the genotypes.

**Figure 6** shows a linkage disequilibrium plot. The referent SNP is rs10934581, because it is the one having the strongest association with serum calcium in CoLaus. Each square represents the position of the SNP on the genome, represented on the abscissa; the number in parenthesis indicates the exact position of the SNP on the genome. The green line represents the *CASR* gene. The blue line represents the recombination rate on the genome (right ordinate axis). The region between two spikes define haplotypes, it means regions of the genome transmitted together during meiosis. The color inside the square represents the value of $r^2$; the red indicates a $r^2$ of 1, the white indicates a $r^2$ of 0. Rs1095681 is strongly correlated with itself and rs11716910 is also strongly correlated to the reference SNP. On the

contrary, rs5008830 and rs2001548 are not correlated with the reference SNP (they are correlated to each other, but this is not represented on this figure. This graphic is consistent with the results depicted in **table 8**.

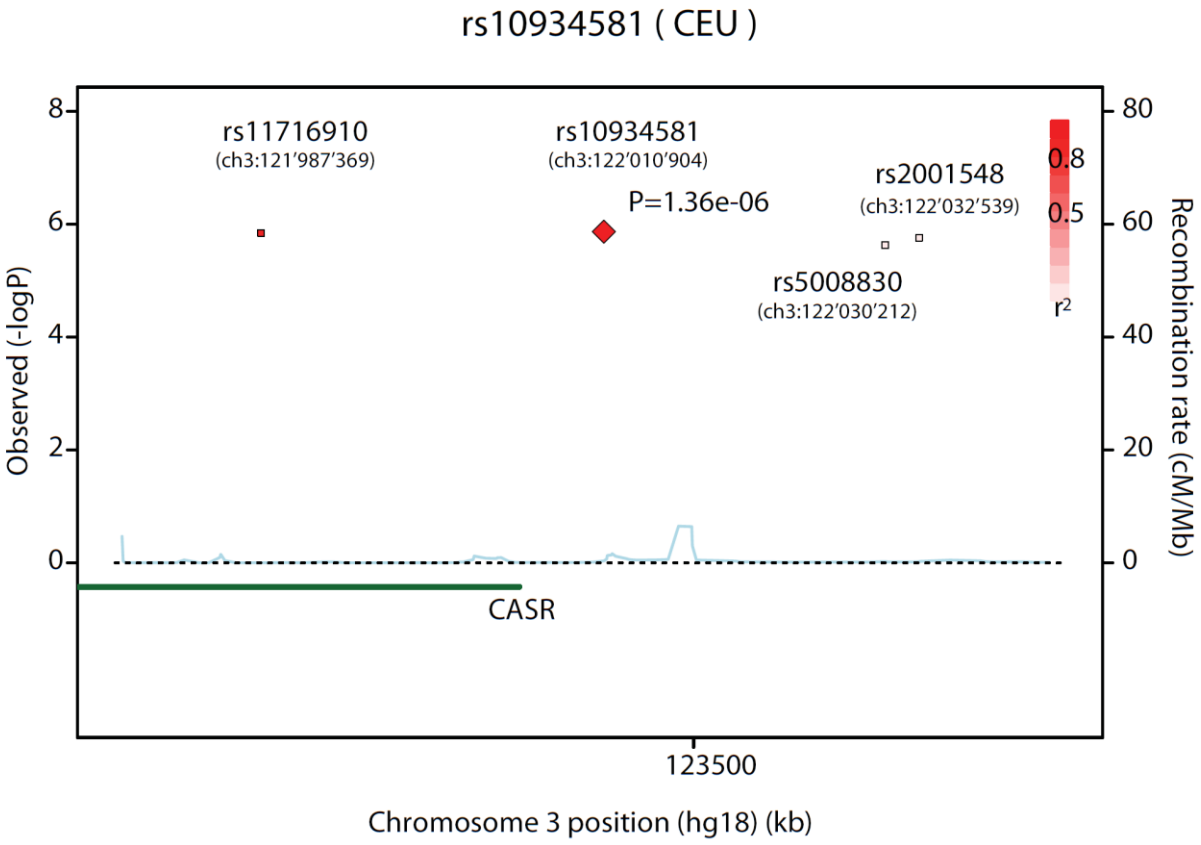


**Figure 6. LD plot for rs10934581, the reference SNP.**

2.4. Association between *CASR* SNPs and hypertension

**Table 14. $X^2$ test for association between SNPs and hypertension.**

| **Rs10934581** | **C/C** | **T/C** | **T/T** | **p-values** |
|---|---|---|---|---|
| **Hypertensive** | 864 (43.86%) | 890 (45.18%) | 216 (10.96%) | |
| **Normotensive** | 1471 (42.48%) | 1548 (44.70%) | 444 (12.82%) | 0.123 |
| | | | | |
| **Rs5008830** | **G/G** | **A/G** | **A/A** | |
| **Hypertensive** | 1289 (66.24%) | 579 (29.75%) | 78 (4.01%) | |
| **Normotensive** | 2261 (65.80%) | 1050 (30.56%) | 125 (3.64%) | 0.686 |

Data are numbers of people in each group, with percentage in brackets. P-values are for the $X^2$ test.

The $X^2$ test used to compare hypertensive and normotensive doesn't show any statistical difference in the genotype distribution across both groups; indeed, the p-values are 0.123 for rs10934581 and 0.686 for rs5008830. The following table (**table 14**) gives the repartition of genotypes for each SNP across hypertensive and normotensive. The last row gives the p-values for the $X^2$ test.

2.5. Association between *CASR* SNPs and systolic and diastolic blood pressure

Despite no statistical association between the selected SNPs and hypertension, the ANOVA test was used to look for differences in the mean systolic and diastolic blood pressures across the three genotypes (**table 15**). As for the previous $X^2$ test, p-values are above 0.3; it means that carrying one genotype or the other leads to no statistical different blood pressure levels. Consequently, there is no association of the selected SNPs with blood pressure levels in CoLaus. The following table gives the values of systolic and diastolic blood pressure for both SNPs and for each genotype.

**Table 15. Systolic and diastolic blood pressure according to the genotypes with ANOVA test results.**

| **Rs10934581** | | **C/C** | **T/C** | **T/T (minor allele)** | **P ANOVA** |
|---|---|---|---|---|---|
| | **SBP** | 128.9 (0.375) | 128.4 (0.358) | 127.9 (0.710) | 0.40 |
| | **DBP** | 79.4 (0.226) | 79.4 (0.218) | 78.9 (0.404) | 0.55 |
| **Rs5008830** | | **G/G** | **A/G** | **A/A (minor allele)** | **P ANOVA** |
| | **SBP** | 128.6 (0.304) | 128.2 (0.444) | 128.2 (1.124) | 0.72 |
| | **DBP** | 79.3 (0.181) | 79.2 (0.267) | 80.2 (0.760) | 0.50 |

Data are values of systolic and diastolic blood pressure in mmHg with standard errors in brackets. The last row gives the p-value from an ANOVA test.

2.6. Stratified analyses

After the $X^2$ test and the ANOVA test, stratification analyses were conducted to look for an association between the SNPs and hypertension in particular subgroups of participants. The SNPs rs10934581 is statistically associated with hypertension in people younger than 55 years old. For the same SNP, there is a trend for an association with hypertension in women and in people with a calcium concentration below 2.21mM ($p = 0.058$ and $p = 0.059$ respectively). The second SNP, rs5008830 is almost associated with hypertension in obese ($BMI \geq 30 kg/m^2$; p-value = 0.067) and in younger ($p = 0.098$). Except for those results, the stratified analysis showed no further association between the SNPs and hypertension (**table 16**).

**Table 16. $X^2$ test for association between SNPs and hypertension in ten subgroups of the CoLaus cohort.**

| Variable | rs10934581 | rs5008830 |
|---|---|---|
| **<55 year** | 0.038* | 0.098 |
| **≥55 year** | 0.855 | 0.723 |
| **Men** | 0.812 | 0.234 |
| **Women** | 0.058 | 0.541 |
| **BMI<30kg/m$^2$** | 0.149 | 0.456 |
| **BMI≥30kg/m$^2$** | 0.615 | 0.067 |
| **Corrected calcium <2.21M** | 0.059 | 0.233 |
| **Corrected calcium ≥2.21mM** | 0.823 | 1.000 |
| **Creatinine <78.1µM** | 0.331 | 0.446 |
| **Creatinine ≥78.1µM** | 0.327 | 0.130 |

Data are p-values from a $X^2$ test.

The **tables 17 to 21** give the systolic and diastolic blood pressure levels for each genotype of both SNPs stratified by age, sex, BMI, corrected calcium and creatinine groups. Despite a statistical association between rs10934581 and hypertension in people below 55 years old, the ANOVA test shows no significant difference in the mean systolic or diastolic blood pressures in the three genotypes as depicted in **table 17**. The second SNPs provides also a slightly higher SBD and DBP in homozygous for the minor allele, but once again, it is not statistically significant.

**Table 17. Association between *CASR* SNPs and blood pressure. Stratification by age.**

| Rs10934581 | | C/C | T/C | T/T | ANOVA |
|---|---|---|---|---|---|
| **SBP** | **<55 years** | 122.3 (0.415) | 122.4 (0.394) | 121.2 (0.735) | 0.34 |
| | **≥55 years** | 136.7 (0.572) | 135.9 (0.562) | 136.2 (1.126) | 0.63 |
| **DBP** | **<55 years** | 78.2 (0.302) | 78.3 (0.289) | 77.8 (0.515) | 0.72 |
| | **≥55 years** | 80.9 (0.336) | 80.9 (0.327) | 80.3 (0.633) | 0.73 |
| **Rs5008830** | | **G/G** | **A/G** | **A/A** | **ANOVA** |
| **SBP** | **<55 years** | 122.0 (0.326) | 122.3 (0.493) | 124.3 (0.320) | 0.21 |
| | **≥55 years** | 136.5 (0.469) | 136.0 (0.694) | 135.7 (1.779) | 0.81 |
| **DBP** | **<55 years** | 78.1 (0.250) | 78.2 (0.357) | 79.9 (1.013) | 0.16 |
| | **≥55 years** | 80.8 (0.273) | 80.6 (0.398) | 80.8 (1.082) | 0.91 |

Data are values of systolic and diastolic blood pressure in mmHg with standard errors in brackets. The last row gives the p-value from an ANOVA test.

Concerning the stratification by sex (see **table 18**), no significant difference across groups appears in this table. Women have a lower SBP and DBP of men regardless of their genotype and no genotype has a significantly higher or lower blood pressure level than another.

**Table 18. Association between *CASR* SNPs and blood pressure. Stratification by sex.**

| **Rs10934581** | | **C/C** | **T/C** | **T/T** | **ANOVA** |
|---|---|---|---|---|---|
| **SBP** | **M** | 132.5 (0.514) | 132.6 (0.481) | 131.7 (0.955) | 0.68 |
| | **F** | 125.7 (0.524) | 124.4 (0.503) | 124.4 (1.004) | 0.18 |
| **DBP** | **M** | 81.4 (0.335) | 81.7 (0.312) | 80.7 ( 0.587) | 0.33 |
| | **F** | 77.7 (0.298) | 77.4 (0.293) | 77.4 (0.545) | 0.64 |
| **Rs5008830** | | **G/G** | **A/G** | **A/A** | **ANOVA** |
| **SBP** | **M** | 132.5 (0.409) | 132.2 (0.602) | 134.0 (1.603) | 0.60 |
| | **F** | 125.2 (0.428) | 124.8 (0.621) | 123.0 (1.399) | 0.48 |
| **DBP** | **M** | 81.3 (0.260) | 81.5 (0.401) | 82.8 (1.086) | 0.36 |
| | **F** | 77.6 (0.245) | 77.3 (0.345) | 77.8 (1.013) | 0.72 |

Data are values of systolic and diastolic blood pressure in mmHg with standard errors in brackets. The last row gives the p-value from an ANOVA test.

People with a BMI above 30 kg/m$^2$ have both systolic and diastolic blood pressures higher than people with a normal weight (**see table 19**). A not statistically significant trend to a lower SBP appears in obese carrying the minor allele A for rs5008830; indeed, homozygous for the major allele have a SBP of 137.5mmHg while heterozygous and homozygous for the minor allele C have a SBP 2.9 and 4.1mmHg lower. This effect is not found for DBP in this subgroup.

**Table 19. Association between *CASR* SNPs and blood pressure. Stratification by BMI.**

| **Rs10934581** | | **C/C** | **T/C** | **T/T** | **ANOVA** |
|---|---|---|---|---|---|
| **SBP** | **BMI < 30kg/m$^2$** | 127.3 (0.401) | 127.0 (0.383) | 126.3 (0.758) | 0.51 |
| | **BMI ≥ 30kg/m$^2$** | 137.4 (0.912) | 135.6 (0.911) | 136.6 (1.737) | 0.37 |
| **DBP** | **BMI < 30kg/m$^2$** | 78.4 (0.243) | 78.6 (0.234) | 77.8 (0.419) | 0.31 |
| | **BMI ≥ 30kg/m$^2$** | 85.0 (0.521) | 84.0 (0.532) | 85.1 (1.081) | 0.37 |
| **Rs5008830** | **BMI < 30kg/m$^2$** | **G/G** | **A/G** | **A/A** | **ANOVA** |
| **SBP** | **BMI < 30kg/m$^2$** | 127.0 (0.323) | 127.1 (0.477) | 126.9 (1.236) | 1.0 |
| | **BMI ≥ 30kg/m$^2$** | 137.5 (0.754) | 134.6 (1.116) | 133.4 (2.537) | 0.37 |
| **DBP** | **BMI < 30kg/m$^2$** | 78.3 (0.193) | 78.4 (0.285) | 79.1 (0.835) | 0.31 |
| | **BMI ≥ 30kg/m$^2$** | 85.0 (0.427) | 83.5 (0.685) | 84.6 (1.652) | 0.37 |

Data are values of systolic and diastolic blood pressure in mmHg with standard errors in brackets. The last row gives the p-value from an ANOVA test.

**Table 20. Association between *CASR* SNPs and blood pressure. Stratification by corrected calcium.**

| **Rs10934581** | | **C/C** | **T/C** | **T/T** | **ANOVA** |
|---|---|---|---|---|---|
| **SBP** | **Calcium < 2.21mM** | 124.0 (0.918) | 124.1 (0.791) | 124.0 (1.349) | 1.0 |
| | **Calcium ≥ 2.21mM** | 129.9 (0.408) | 129.5 (0.398) | 129.1 (0.822) | 0.64 |
| **DBP** | **Calcium < 2.21mM** | 77.2 (0.559) | 77.6 (0.500) | 78.4 (0.822) | 0.54 |
| | **Calcium ≥ 2.21mM** | 79.8 (0.246) | 79.9 (0.241) | 79.1 (0.464) | 0.29 |
| **Rs5008830** | | **G/G** | **A/G** | **A/A** | **ANOVA** |
| **SBP** | **Calcium < 2.21mM** | 124.7 (0.678) | 122.3 (0.971) | 124.2 (3.462) | 0.15 |
| | **Calcium ≥ 2.21mM** | 129.7 (0.337) | 129.5 (0.490) | 128.9 (1.180) | 0.82 |
| **DBP** | **Calcium < 2.21mM** | 77.8 (0.415) | 76.9 (0.630) | 78.0 (2.206) | 0.52 |
| | **Calcium ≥ 2.21mM** | 79.8 (0.200) | 79.7 (0.293) | 80.5 (0.808) | 0.63 |

Data are values of systolic and diastolic blood pressure in mmHg with standard errors in brackets. The last row gives the p-value from an ANOVA test.

**Table 21. Association between *CASR* SNPs and blood pressure. Stratification by creatinine.**

| **Rs10934581** | | **C/C** | **T/C** | **T/T** | **ANOVA** |
|---|---|---|---|---|---|
| **SBP** | **Creat < 78.1µM** | 126.6 (0.530) | 125.9 (0.513) | 126.1 (1.036) | 0.60 |
| | **Creat ≥ 78.1µM** | 131.1 (0.524) | 130.8 (0.492) | 129.6 (0.962) | 0.38 |
| **DBP** | **Creat < 78.1µM** | 78.3 (0.315) | 78.1 (0.300) | 78.3 (0.577) | 0.87 |
| | **Creat ≥ 78.1µM** | 80.6 (0.321) | 80.7 (0.311) | 79.5 (0.565) | 0.17 |
| **Rs5008830** | | **G/G** | **A/G** | **A/A** | **ANOVA** |
| **SBP** | **Creat < 78.1µM** | 126.6 (0.441) | 125.6 (0.615) | 124.0 (1.389) | 0.16 |
| | **Creat ≥ 78.1µM** | 130.6 (0.414) | 131.0 (0.626) | 133.1 (1.685) | 0.34 |
| **DBP** | **Creat < 78.1µM** | 78.4 (0.256) | 77.7 (0.357) | 78.5 (1.043) | 0.30 |
| | **Creat ≥ 78.1µM** | 80.3 (0.254) | 80.9 (0.391) | 82.1 (1.081) | 0.14 |

Data are values of systolic and diastolic blood pressure in mmHg with standard errors in brackets. The last row gives the p-value from an ANOVA test.

## 3. Discussion

This work analyzed the association between two SNPs in or around the *CASR* gene (rs10934581 and rs5008830) and hypertension in CoLaus, a population-based study. The results showed no association of those SNPs with hypertension or systolic/diastolic blood pressure levels. In the stratified analysis, rs10934581 was associated with hypertension in people younger than 55 years old, but other results were not statistically significant. Nevertheless, a trend can be detected for an association of this SNP with hypertension in women and in people with a calcium concentration below 2.21mM ($p = 0.058$ and $p = 0.059$ respectively). The second SNP, rs5008830, is almost associated with hypertension in

obese participants (BMI ≥ 30kg/m$^2$; p-value = 0.067) and in younger (< 55 years; p = 0.098), but once again, the results are not statistically significant.

Numerous candidate gene studies have been conducted to explore genetics of hypertension. So far only three candidate gene studies focused on the association of *CASR* variants with hypertension and/or blood pressure.

In 2000, Pratt and al. (33) explored the link between 3 *CASR* polymorphisms known to be associated with serum calcium (25) (A986S, G990R and Q1011E) and sodium excretion and blood pressure. Even if CASR is a channel implicated in urinary calcium excretion, they explored urinary sodium excretion, because Fuleihan (34) and al. showed that an increase in serum calcium leads to an increased urinary sodium excretion. They studied urinary sodium excretion in 191 black and 282 white children (these children were chosen because of their lack of age-related cardiovascular disease, renal disease, diabetes mellitus or medication intake, that may be confounding factors for the sodium excretion) and blood pressure in normotensive and hypertensive white (379 people) and black (455 people) adults with a BMI less than 32 kg/m$^2$. They found no association of these three SNPs with sodium balance or with blood pressure. However, they found that the Q1011 allele was more prevalent in Blacks than in Whites and that the polymorphism Q1011E was marginally associated with higher SBP and DBP in black people (except after adjustment for multiple comparisons).
One limitation of this study may be the low allele frequency of the studied SNPs. In CoLaus, the MAF of the SNPs studied in this work is 18% and 34%. In Pratt's cohort, the highest MAF is 16.5% for Q1011, but only 3 to 13% for the other alleles. As this sample size is small, especially that of the children subgroup, it is difficult to detect a small effect of genetic polymorphisms on blood pressure. The only effect that was demonstrated concerned the allele with the highest prevalence in the biggest subgroup (black people were more numerous in this study than white people). In CoLaus, both the MAF and the number of participants are higher than in Pratt's study, but despite this, no association of *CASR* SNPs with blood pressure was found.

However, in 2008, Tobin and al.(6) evaluated the effect of common variants in five genes (*CASR, NR3C2, SCNN1B, SCNN1G* and *KCNJ1*) on blood pressure in a family-based study including 2037 Europeans from 520 families. Their first outcome was 24-hour ambulatory blood pressure (in CoLaus, the ambulatory blood pressure is not available). Their main result was a strong association of SNPs in the *KCNJ1* with both SBP and DBP; as loss-of-function mutations in this gene cause type II Bartter's syndrome, a monogenic form of hypotension, one could expect that activating mutation have the opposite effect on blood pressure and this was verified in this study. In addition, Tobin and al. discovered that variants in the *CASR* gene were associated with 24-hour SBP and DBP (6). They used

16 SNPs of the *CASR*[12]; one of them was rs11716910, as in this work, but it was not statistically associated with mean 24h, mean daytime, mean night-time and clinic SBP and DBP, nor with 24h urinary sodium, potassium and calcium (p-values were above 0.5). However, other SNPs in the *CASR* were associated with those variables[13]. This study shows the importance of a large sample size and the quality of the phenotype (i.e. 24-hour blood pressure) in studies aiming at unraveling the genetic determinants of hypertension.

In 2009, Jung and al. (36) (Jung and Pratt, who published the study described above, belong to the same team) included, in a longitudinal study, the same black adolescents previously studied by Pratt in 2000 to look for an association between fifteen *CASR* SNPs and hypertension. Only the black participants were followed since 2000, because they tend to have higher sodium retention, lower calcium excretion as well as a blood pressure more sensitive to a diet rich in calcium or salt than Whites. All these elements suggest that an association between genetic variants and hypertension may be easier to detect in this ethnic group. This population-based study found three SNPs in strong linkage disequilibrium (rs6438712, rs4678172 and rs937626) to be associated with systolic blood pressure. This second study shows that even with a relatively small sample, a better gene coverage (fifteen versus three SNPs) may be sufficient to detect significant associations. Despite this positive result, Jung and al. surprisingly failed to mention their previous negative result in the bibliography and did not clearly say that the same participants were included in both studies. But in this second study, Jung et al added participants from a family-based study: 123 parents of the adolescents were included in the sample. Rs4678172 was associated with blood pressure in the family-based and population-based studies.

Jung and al. also examined the ethnic difference in urinary excretion of calcium; their previous study examined the sodium excretion, but obtained negative results. This cross-sectional study included 106 African-Americans and European-Americans subjects and their parents (for 88 subjects) and once again, a population-based and a family-based analysis was conducted. Six SNPs were associated with calcium excretion, including four SNPs associated with blood pressure in both analyses. The alleles associated with higher blood pressure were associated with a lower urinary calcium excretion. The effect-size of the SNPs was calculated to be 2 mmHg in the population-based study and 3 mmHg in the family-based study, which can be considered to be quite large.

These results suggest that the *CASR* gene might play a role in blood pressure control in the population. The results by Jung et al are consistent with the hypothesis that CASR influences the function of

[12] rs7648041, rs1048213, rs6762782, rs1973490, rs17203502, rs6783556, rs13320637, rs1354162, rs3863977, rs7647446, rs2036399, rs11716910, rs13095172, rs1801725, rs10190 and rs7621124.
[13] See annex 3

NKCC2 (CASR inhibits NKCC2). When the CASR is less activated, as evidenced by a lower excretion of calcium, the blood pressure is higher. Consequently, a loss-of-function mutation in the *CASR* may lead to higher sodium reabsorption and therefore to a higher blood pressure level. As described in Jung's article, this effect is particularly evident in African-Americans, whose NKCC2 activity is greater than in European-Americans. This ethnic difference in a channel activity suggests that the prevention and the treatment of hypertension should not be the same for everyone; each group and at term each person may need to receive a targeted and individual support.

As depicted in the three candidate gene studies previously described, positive results demonstrating an association between gene polymorphisms and a multifactorial disease such as hypertension are difficult to obtain and to replicate across studies. The CoLaus study is a large population-based study, yet, genome-wide association studies (GWAS) showed that a study is not powerful enough if the number of participants is less than 20,000. The difference between GWAS and candidate gene studies is that GWAS are hypothesis free.

As depicted in the first part of this work, three GWAs were conducted since 2007 with the aim of looking for SNPs in the human genome associated with blood pressure. The WTCCC in 2007 (38) did not find any result and the other two consortia (Global BPgen (12) and CHARGE (13) in 2009) detected about 20 SNPs associated with hypertension, high SBP or DBP, but none of those SNPs was localized in classical genes implicated in blood pressure control. Those results prove that GWA is a good method to detect genetic variants associated with common diseases such as hypertension, but they also show that important means are necessary in term of participants, researchers, time and money. A limitation of these studies is that they do not take into account the interactions between genes and the influence of environmental conditions in the development of hypertension. The locus containing the *CASR* gene was not found to be associated with blood pressure or hypertension in these GWAS.

This CoLaus study has limitations that will be described below. However, to limit and detect confounding factors of effect modifiers, stratified analyses were conducted. Confounding factors are risk factors for a disease or a condition (here hypertension, SBP and DBP) other than the one studied (in our case: SNPs in the *CASR*) that are associated both with the genetic risk factor of interest and the outcome of interest (here blood pressure or hypertension). An effect modifier is a factor interacting with the studied factor; both factors may have the same effect on the outcome (here: blood pressure level and hypertension) or they can have an opposite effect. If for example, sex modifies the effect of one SNP on blood pressure in the sense that a specific risk allele is associated with lower blood pressure in men and higher blood pressure in women, an analysis combining men and women may fail to detect the association of this SNP with blood pressure. A stratified analysis will allow detecting

such potential effect modification.

I decided to stratify for five variables. The first one was age. Blood pressure increases with age because 1) with age, there is a structural modification in the wall of arteries leading to a loss of elasticity and a stiffening of arteries and 2) people become more sensitive to salt with age; hypertension itself sensitizes to salt; it is a vicious circle. Participants were divided in two subgroups, above and below 55 years old; this age was chosen as a limit, because diastolic blood pressure increases until the age of 55 and decreases after the age-range of 55 to 60 years old. The second variable for stratification was sex. Men generally have a higher blood pressure level than women (it is also the case in CoLaus). This is due to the protective action of estrogens against cardiovascular events; postmenopausal women do not benefit from this protective effect anymore and reach the same risk as men. Thirdly, BMI was chosen as a variable for stratification. The limit of 30 $kg/m^2$, that is the threshold to define obesity, was used to separate participants in two subgroups, because 1) obesity and hypertension are two components of the metabolic syndrome; 2) weight excess leads to an increase in blood pressure levels; 3) there is a higher proportion of hypertensive among obese than in the general population and 4) obesity is also known to influence blood pressure sensitivity to salt. The fourth variable included in the stratified analysis was corrected serum calcium (corrected calcium concentration reflects the quantity of ionized calcium, which is the physiologically active calcium). This variable was used because of the known association of *CASR* with serum calcium and the potential of role of calcium in hypertension. The last variable included in the stratification was serum creatinine. Indeed, renal function and blood pressure level closely interaction with each other. In case of renal failure, one part of the kidney is not functional anymore and there is a compensatory hypertrophy of the remaining functional nephrons. The renin-angiotensin-aldosterone system (RAAS) is activated; this leads to a renal retention of sodium and water by the kidney, with finally an elevation of the blood pressure level. The RAAS also leads to a vasoconstriction of arteries. Both these elements contribute to a higher blood pressure level. Hypertension in turn may worsen renal function and is an important risk factor for chronic kidney disease. Atherosclerosis in afferent and efferent arterioles of the juxtaglomerular apparatus is the most common lesions in hypertension; they lead to a diminution of glomerular filtration rate and tubular dysfunction.

Despite the stratification, some limitations in the CoLaus remain that may partially explain the absence of association between the selected *CASR* SNPs and hypertension or blood pressure. Firstly, this study is probably underpowered. The CoLaus study is a single-centre study; even if 6,188 subjects already represent a large sample, it is not enough to detect the small effect that a single SNP may have on blood pressure level. The effect sizes of the SNPs discovered by Jung were of 2 to 3 mmHg on the blood pressure. This is quite large compared to the effect sizes discovered in recently conducted GWAS (< 1 mm Hg).

Secondly, in the CoLaus study, the urinary calcium and sodium excretion were not measured. These phenotypes are extremely important considering the role of CASR in urinary calcium excretion and the natriuretic effect of extracellular calcium (34). Also, calcium binding to CASR inhibits the NKCC2 in the TAL, which decreases renal tubular sodium reabsorption (39). The value of urinary sodium is also important because salt balance is correlated to blood pressure; when the value of serum sodium would be available, the fractional excretion of sodium could be calculated to evaluate the function of the kidney.

Thirdly, in this work, only four SNPs were chosen and among them, only two were selected for the analyses. One of the SNP was excluded from further analyses because it did not follow the Hardy-Weinberg proportions (p-values for the $X^2$ test = 0.048) and a second because of a strong correlation with another SNP. The gene coverage was very poor which strongly limited the ability to detect any association. A complete and more extensive analysis of the association of *CASR* variants with hypertension and blood pressure in CoLaus is beyond the scope of this master's thesis because of the complexity of the statistical analyses. The following step after this work would be to extend the analysis to all SNPs located within and around the *CASR* gene that are available in CoLaus and to conduct haplotype-based analyses as well as potential gene-gene interactions.

One technical aspect also contributes to the insufficient gene coverage in this work. Only SNPs present on the Affymetrix 500K chip were available. This chip does not specifically target genes and many genes are therefore not sufficiently covered. SNPs found on the Affymetrix chip only represent a tiny subset of currently known SNPs on the human genome. The HapMap database contains about 15 millions SNPs dispersed in the human genome. Those SNPs are markers of the heterogeneity of the human genome, but they are not specifically located in genes. The SNPs available for the *CASR* gene were selected by chance, that is without consideration of their position in the gene or even around the gene (remember that a marker of a disease can segregate even if it is not located in the gene responsible for the disease), nor of their function. For this work, four SNPs were selected: rs10934581, rs5008830, rs2001548 and rs11716910. Among them, only the last one (rs11716910) is located in the *CASR* gene, in an intron. The others are not located in the gene itself but in a region close to it. Because of the low number of SNPs in this work and the fact that they may not adequately cover the region of interest around the *CASR* gene, the present work should be considered as exploratory and the negative results obtained so far do not provide sufficient evidence to exclude an implication of *CASR* in hypertension or blood pressure in CoLaus. More extensive analyses are needed to further explore the potential role of *CASR* SNPs in hypertension and blood pressure in CoLaus.

Despite the large number of participants, a GWA approach such as by using the Affymetrix chip in a population-based study like CoLaus is not appropriate to detect the role of rare variants. Genome

sequencing approaches that allow identifying all variants within and around a specific gene should help better understand the role of rare variants in common complex traits like hypertension.

Fourthly, another limitation of this study is that the blood pressure phenotype is based on the mean of two out of three measurements. As described in the methodology, the blood pressure was measured three times on the left arm after a ten-minute rest in a sitting position with an automated oscillometric sphygmomanometer (Omron). However, following the guidelines, a diagnosis of hypertension cannot be made at a single clinical visit. Ideally, blood pressure should be measured during several visits, which was not the case for CoLaus. The second remark is that even if the measure was taken three times, it represents only a single value in time and cannot accurately reflect the circadian blood pressure pattern. The fact that the measure was not made at home is also a source of variation, because it is known that blood pressure may be higher at the doctor's office than at home, a phenomenon called the white coat effect. Ideally, it would be good to have a 24-hour monitoring of the blood pressure like in Tobin's study or at least two groups of three measures, one at the place of the study and the other at home. Note also that the blood pressure used for hypertensive is not the values that were measured during the consultation. Indeed, as hypertensive people are taking medications, their measured blood pressure is underestimated. Because of that, 15 mmHg and 10 mmHg are added to the measured SBP and DBP respectively.

Finally, one may remind that *CASR* may interact with other genes or environment. The previous review of the literature showed that the CASR is present in several tissues. In the kidney only, it influences the function of many transporters, including NKCC2 and ROMK, both channel implicated in Bartter's syndrome, a monogenic form of hypotension. However, CASR is firstly a calcium sensing receptor and may interfere with other components of calcium homeostasis, including vitamin D and PTH. This hypothesis should be explored, because low vitamin D levels have been associated with higher incidence of hypertension (3). Thus, vitamin D, PTH and CASR may interact in the kidney and have repercussion on sodium balance. Maillard and al. also have demonstrated that the CASR in the juxtaglomerular apparatus contributes to the regulation of rennin release; this suggests a potential interaction between *CASR* and other components of the rennin-angiotensin-aldosterone system. Other gene interactions may exist; including the one between *CASR* and *SCL12A1/2* (type I and V Bartter's syndrome are due to monogenic mutations in those genes).

Despite an absence of association of selected *CASR* variants with hypertension and blood pressure in the CoLaus study, *CASR* remains a good candidate gene for hypertension in humans. The first reason to be interested in *CASR* as a candidate gene for hypertension is that 1) SNPs in the *CASR* were found to be associated with hypertension in Jung's study in African-Americans and 2) variants in the *CASR* gene were also associated with 24-hour systolic and diastolic blood pressure in Tobin's study. The

second good reason to be interested in *CASR* is its role in type V Bartter's syndrome. Unlike essential hypertension, this autosomal dominant disease manifests itself at a very young age and is a very rare condition. People present with hypotension and hypercalciuria, while Jung's variants are associated with hypertension and hypocalciuria. It reinforces the idea that loss-of-function mutations in *CASR* may explain hypertension in the general population. Even if known loss-of-function mutations cause FBHH, NSHPT and FIHP, disease in which blood pressure is normal, Jung's results suggest that other variants may be associated with SBP or DBP.

A third reason to further explore the association of *CASR* variants with blood pressure is the potential interaction with other genes. Tobin's study showed that activating mutations in *KCNJ1*, the gene implicated in type II Bartter's syndrome, were associated with higher 24-hours SBP and DBP. We already know that the CASR in the TAL inhibits the ROMK; it means that both channels interact with each other. Consequently, one may hypothesize that an activating mutation in the *CASR* gene may lead to a lower activity of the ROMK and to a higher blood pressure level.

**Perspectives**

Further studies with other data and more people may try once again to discover an association between CASR and hypertension. A random subset of CoLaus participants has undergone 24-hour blood pressure monitoring (Hercules study). Hercules participants also have data on 24-hour urinary sodium and calcium excretion. It will be of major interest to explore the associations of available *CASR* variants with these phenotypes in Hercules.

The goals of the studies exploring the influence of genes on blood pressure are to get better understanding of the mechanisms of essential hypertension. The discovery of the implication of genes in the development of hypertension may eventually have clinical applications. First of all, knowing that a gene is an important risk factor to develop hypertension may permit to screen for people at risk in the general population and to make targeted prevention. The actual guidelines used in the PMU in Lausanne say that the doctor has to measure the blood pressure of every patient every two years from the age of eighteen. The goal of screening for hypertension is to make prevention. If the genotype of the patients is known, the prevention could target people that are especially at risk of developing hypertension. Even if the prevention for cardiovascular risk factor is one task of all physicians, time is sometimes lacking and targeting people at high risk of developing hypertension would be a good way to be more effective. The final goal of such studies is not only to be able to detect hypertension, but also to treat patients.

Drugs currently used to treat hypertension act only on a few systems: adrenergic receptors (alpha and beta-antagonists, alpha2-agonists), calcium receptors (calcium channel blockers), renin-angiotensin system (angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, aldosterone

antagonists) and water-salt balance (diuretics). Discovering new pathways implicated in blood pressure regulation may permit to develop new molecules to treat patients suffering from hypertension and to develop individualized treatments.

Genetic tests aiming at detecting people who are at high risk of making an adverse drug reaction are already marketed and can be used in clinics in specific cases (for example, Asians that have to receive carbamazepine should be genotyped for HLAB*1502 because this genetic variant is associated with a high risk of developing a Lyell syndrome while taking this drug). Such genetic tests could be developed to detect people carrying mutations strongly associated with antihypertensive drug-related side effects.

However, we do not know if genetics test may be useful to increase the rate of patients treated for hypertension. Indeed, 35.9% of hypertensive are taking a medication in CoLaus. Several causes may explain this low rate of treatment: 1) the screening may be insufficient, 2) physicians may not treat every people presenting a too high blood pressure level and/or 3) patients compliance to treatment may be low. Perhaps, knowing the genome of his patients and knowing that he may have in individualized treatment may be a further argument to convince a physician to make prevention, to screen and to treat his patients.

**4. Conclusion**

Even if genetic determinants play a role in hypertension, genes do not act alone and interactions are possible between genes but also between genes and environmental factors such as diet. Hypertension is a multifactorial disease with an important environmental component that researchers and physicians working on hypertension cannot ignore. When one variant is shown to be associated with hypertension, the effect of this single variant on the blood pressure is often very weak (<1 mmHg). For all these reasons, studying the interaction between genes and hypertension is a very long work that demands patience, perseverance and time, together with high financial and human resources.

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# IV. Annexes

## Annex 1 : References of figures

Figure 1. GPCR topology of the CASR.

http://www.casrdb.mcgill.ca/?Topic=CasrGraph&v=new 12.11.2009, 2:27pm

Figure 2. CASR signaling in the parathyroid gland

http://www.studentconsult.com/content/figure.cfm?mediaISBN=0721632564&imagebodyID=F051007&ImageSeqNo=77746150&screenWidth=1400&screenHeight=1050

Figure 3. Reabsorption of calcium in the proximal tubule

http://www.studentconsult.com/content/figure.cfm?mediaISBN=0721632564&imagebodyID=F035016&ImageSeqNo=77569850&screenWidth=1400&screenHeight=1050

Figure 4. Reabsorption of calcium in the TAL

http://www.studentconsult.com/content/figure.cfm?mediaISBN=0721632564&imagebodyID=F035016&ImageSeqNo=77569900&screenWidth=1400&screenHeight=1050

Figure 5. Reabsorption of calcium in the DCT

http://www.studentconsult.com/content/figure.cfm?mediaISBN=0721632564&imagebodyID=F035016&ImageSeqNo=77569950&screenWidth=1400&screenHeight=1050

## Annex 2 : References from articles coming from the website UpToDate

« Disorders of the calcium-sensing receptor : familial hypocalciuric hypercalcemia and autosomal dominant hypercalcemia »

http://www.uptodate.com/online/content/topic.do?topicKey=calcium/6524&selectedTitle=1%7E150&source=search_result 22.04.2010, 14 :04

« Overview of vitamin D »

http://www.uptodate.com/online/content/topic.do?topicKey=bone_dis/14700&selectedTitle=1%7E150&source=search_result 22.04.2010, 14 :04

« Hormonal regulation of calcium and phosphate balance »

http://www.uptodate.com/online/content/topic.do?topicKey=ren_phys/9023&selectedTitle=1%7E150&source=search_result 22.04.2010, 14 :04

« Diuretics and calcium balance »

http://www.uptodate.com/online/content/topic.do?topicKey=fldlytes/26574&selectedTitle=1%7E90&source=search_result

22.04.2010, 14 :05

« Bartter's and Gitelman's syndromes »

http://www.uptodate.com/online/content/topic.do?topicKey=fldlytes/27746&selectedTitle=1%7E25&source=search_result

22.04.2010, 14 :06

« Cell model for the loop of Henle transport »

http://www.uptodate.com/online/content/topic.do?topicKey=ren_phys/6189&selectedTitle=1%7E150&source=search_result

22.04.2010, 14 :06

« Overview of hypertension in adults »

http://www.uptodate.com/online/content/topic.do?topicKey=hyperten/23203&selectedTitle=1%7E150&source=search_result

22.04.2010, 14 :07

**Annex 3 : Association of SNPs of the *CASR* with mean night SBP, mean nigh DBP, mean 24-hours SBP, mean 24-hours DBP, and 24-hours urinary calcium in Tobin's study**

| SNP | Allele | Variable | p-value |
|---|---|---|---|
| **rs7648041** | C/T | meannightSBP | 0.047 |
| **rs6762782** | G/A | meannightSBP | 0.013 |
| **rs6762782** | G/A | meannightDBP | 0.011 |
| **rs1973490** | A/T | mean24SBP | 0.031 |
| **rs1973490** | A/T | mean24DBP | 0.02 |
| **rs1973490** | A/T | meannightSBP | 0.0062 |
| **rs1973490** | A/T | meannightDBP | 0.0074 |
| **rs17203502** | A/G | meannightSBP | 0.024 |
| **rs6783556** | A/G | meannightSBP | 0.031 |
| **rs10190** | C/T | 24h urinary calcium | 0.048 |

P-values are for the $X^2$ test.

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