Progression of Mineral Ion Abnormalities in Patients with Jansen's Metaphyseal Chondrodysplasia

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Short title: Mineral Ion Abnormalities in Jansen's Disease

Précis:

Jansen metaphyseal chondrodysplasia is caused by heterozygous activating PTH/PTHrP receptor mutations that lead to mineral ion abnormalities, delayed chondrocyte differentiation, and short stature

ABSTRACT

Context: Five different activating PTH/PTHrP receptor (PTHR1) mutations have been reported as causes of Jansen metaphyseal chondrodysplasia (JMC), a rare disorder characterized by severe growth plate abnormalities and PTH-independent hypercalcemia.

Objectives: Assess the natural history of clinical and laboratory findings in twenty-four JMC patients and characterize the disease-causing mutant receptors *in vitro*.

Patients and Methods: The H223R mutation occurred in 18 patients. T410P, I458R and I458K each occurred in single cases; T410R was present in a father and his two sons. Laboratory records were analyzed individually and in aggregate.

Results: Postnatal calcium levels were normal in most patients, but elevated between 0.15-10 years (11.8±1.37 mg/dL) and tended to normalize in adults (10.0±1.03 mg/dL). Mean phosphate levels were at the lower end of the age-specific normal ranges. Urinary calcium/creatinine (mg/mg) was consistently elevated (children: 0.80±0.40; adults: 0.28±0.19). Adult heights were well below the 3rd percentile for all patients, except for those with the T410R mutation. Most JMC patients had undergone orthopedic surgical procedures, most had nephrocalcinosis, two had advanced chronic kidney disease. The five PTHR1 mutants showed varying degrees of constitutive and PTH-stimulated cAMP signaling activity when expressed in HEK293 reporter cells. The inverse agonist [L^{11},dW^{12},W^{23},Y^{36}]PTHrP(7-36) reduced basal cAMP signaling for each PTHR1 mutant.

Conclusions: Except for T410R, the other PTHR1 mutations were associated with indistinguishable mineral ion abnormalities and cause similarly severe growth impairment. Hypercalciuria persisted into adulthood. An inverse agonist ligand effectively reduced *in vitro* PTH-independent cAMP formation at all five PTHR1 mutants, suggesting a potential path towards therapy.

INTRODUCTION

The PTH/PTHrP receptor (PTHR1) mediates the actions of two peptides, parathyroid hormone (PTH) and PTH-related peptide (PTHrP), which stimulate at least two signaling pathways, cAMP/PKA and Ca^{2+}/IP3/PKC. The PTHR1, a class B G protein-coupled receptor (GPCR), is abundantly expressed in kidney and bone, and in the metaphyseal growth plates (1). In growth plate chondrocytes, activation of the PTHR1 by PTHrP slows the differentiation of chondrocytes, thus contributing importantly to normal bone growth and elongation (2). In bone, activation of the PTHR1 by PTH directly affects osteoblast and osteocyte activity, and indirectly affects, through the RANK/RANKL system, osteoclast maturation and activity. In distal renal tubules, the PTHR1 mediates the PTH-dependent reabsorption of calcium, while in the proximal tubules it enhances excretion of phosphate and the expression of 1α-hydroxylase (3).

Jansen metaphyseal chondrodysplasia (JMC) is a rare autosomal dominant disease caused by heterozygous, activating PTHR1 mutations (4-6). Thus far, five different PTHR1 mutations affecting one of three different amino acid residues have been identified in JMC patients; these mutations, H223R, T410P/R, and I458K/R, are each located at the intracellular end of a transmembrane helices, namely 2, 6, and 7, respectively (7). The constitutive activity of the PTHR1 mutants slows chondrocyte maturation leading to marked growth plate abnormalities that resemble severe rachitic changes (8, 9). In addition to short stature and bowing of long-bones, JMC patients often exhibit micrognathia, hypertelorism, high-arched palate, delayed tooth eruption or impaction, and premature closure of cranial sutures. However, this information is based on anecdotal reports, as a comprehensive natural history profile of JMC has yet to be established (7, 10-16).

Prominent laboratory abnormalities reported for JMC patients include severe PTH- and PTHrP-independent hypercalcemia and hypophosphatemia that are associated with high rates of bone turnover, cortical thinning, and excessive hypomineralized osteoid (14). Severe metaphyseal changes associated with life-long hypercalcemia were thought to be the hallmarks of JMC (7, 11, 13). However, recent reports revealed that some

patients, diagnosed radiographically and genetically with JMC, did not show overt hypercalcemia or hypophosphatemia (13, 17). It is thus currently uncertain as to the extent that radiographic, height, and biochemical abnormalities in JMC can vary due, for example, to patient age and/or type of PTHR1 mutation. In addition, even in the absence of obvious hypercalcemia, urinary calcium excretion may be elevated. Patients affected by JMC can thus be at risk of developing nephrocalcinosis and possibly impaired renal function.

The purpose of the current study was, therefore, to assess the natural history and long-term outcome of multiple patients with documented, disease-causing PTHR1 mutations. We report blood and urinary calcium levels in newborns, children, and adults affected by JMC; adult heights, need for surgical intervention, and other biochemical abnormalities and renal function are also assessed. In addition, we characterize the different JMC-causing PTHR1 variants in cell-based functional assays and investigate *in vitro* their response to a PTH agonist and a PTHrP-based inverse agonist ligand.

SUBJECTS AND METHODS

Patients and data collection

Clinical and laboratory information of previously reported patients was obtained from earlier publications (5, 6, 10-19). No additional patients with a confirmed molecular defect were identified by searching PubMed (Public/Publisher MEDLINE; electronic database on September 27, 2017) using the query "Jansen type metaphyseal chondrodysplasia" [MeSH Terms] OR "Jansen metaphyseal chondrodysplasia" [All Fields]). Whenever possible, follow-up data were obtained from the primary care physician or specialist involved in the care of the patient. In addition, we collected clinical and laboratory information for five patients not previously reported, for whom a disease-causing genetic PTHR1 mutation was identified. Laboratory data are listed according to four age groups; birth until the age of 1.5 months, 0.15-10 years, 17-38 years, and above 49 years. Furthermore, we were able to obtain the final adult height for a subset of 13 patients, as well as information on renal function and calcifications, major skeletal abnormalities, use of bisphosphonates, and surgical interventions. Z-scores for height in children and adults were calculated based on the data from WHO Child Growth Standard, National Health and Nutrition Survey (NHANES), and CDC/National Center for Health Statistics.

Case reports

As examples of the natural course of laboratory abnormalities in Jansen's disease, findings are presented for three previously unreported patients, H223R-15, H223R-16, and H223R-17. Laboratory findings as well as major radiographic and physical abnormalities are also provided for two other unreported patients, H223R-9 and H223R-18 (**Suppl. Table 1**). Patients H223R-4, H223R-13, H223R-14, T410R-2, and T410R-3 each inherited the PTHR1 mutation from an affected parent; all other JMC patients have healthy parents, suggesting that their PTH1R mutation occurred *de novo*.

Patient H223R-15

This four-year-old boy, the first child of healthy parents, presented at birth with breathing difficulties due to micrognathia and bilateral choanal stenosis. He was noted to have hypertelorism, an elongated and high arched palate, downsloping palpebral fissures, and large open fontanelles with widely spaced sagittal sutures, and

palpable rachitic rosary. Investigations in the neonatal period showed serum calcium levels at the upper end of normal (9.64-11.4 mg/dL), with mildly decreased serum phosphate (1.62 mmol/L, normal range at this age: 1.8-3.0) and low serum PTH (12 pg/mL; normal range at this age: 20-95). Over the subsequent months his serum calcium increased (see **Fig. 1**; green filled circles), with associated hypercalciuria, and elevated serum alkaline phosphatase activity, elevated serum $1,25(OH)_2$ vitamin D levels (101 pg/mL, range: 63-136; normal range: 19-76), and progressive suppression of PTH concentration to less than 1 pg/mL. His skeletal survey showed markedly abnormal bones with typical JMC features; the H223R mutation was identified at seven months of age. Serial renal ultrasound examinations, performed during infancy to investigate persistent hypertension, revealed nephrocalcinosis by eight months of age. His hypertension resolved without treatment.

Patient H223R-17

This 25-year-old female was recognized to have abnormal long bone radiographic features on the first day of life; hypercalcemia was noted on day 5. A diagnosis of JMC was made on the basis of clinical, radiographic and biochemical findings at the age of four months. Medical interventions included a low calcium and low salt diet, as well as oral phosphate supplementation for much of her childhood. Her early growth was slow with lengths/heights below the 3rd percentile and further slowing was noted at 3 years of age. She had severe and recurrent alignment abnormalities of her legs (primarily varus deformity and anterior bowing of both the tibiae and femora); multiple osteotomies of both tibiae and both femora were performed between ages of 2.5 and 14 years (at 2.5, 5, 7, 10, and 14 years). Progressive kyphoscoliosis required posterior spinal fusion from T2 to L3 at age 11 years. Her maximal adult height is 116.9 cm. Most recent laboratory studies showed a total serum calcium level of 10.5 mg/dL (upper end of normal) with suppressed PTH (<4 pg/ml). Serum phosphate was at the lower end of the normal range (0.81 mmol/L) and the $1,25(OH)_2$ vitamin D level was 70.1 pg/mL, which is at the upper end of the normal range, although the 25 vitamin D level was only 13 ng/mL (i.e. well below the recommended level of 32 ng/ml). The serum creatinine was 0.39 mg/dL, which yields, based on the Schwartz formula (20, 21), a calculated glomerular filtration rate of 108.9 mL/min/1.73 m^2. Time course of her serum levels from infancy until adulthood are

shown in **Fig. 1** (red open circles) and in **Suppl. Fig. 1** alone with urinary calcium:creatinine ratios; note that the serum calcium level was extremely elevated throughout childhood, but decreased to the upper end of the normal range during adulthood; nevertheless, hypercalciuria and an elevated urinary calcium/creatinine ratios persisted. Medullary nephrocalcinosis was documented in early childhood.

Patient H223R-16

The 56-year-old male had reached a maximal adult height of 133 cm. At that age, his laboratory studies revealed a normal serum calcium level (9.4 mg/dL) with an elevated PTH (312 pg/ml) and a slightly elevated serum phosphate level (1.55 mmol/L), i.e. laboratory findings not typically observed in Jansen's disease. However, his serum creatinine was abnormal at 4.04 mg/dL and the estimated glomerular filtration rate was only 22 mL/min/1.73 m^2, as calculated by the Schwartz formula. A progressive decline in renal function had been noted since his late thirties (**Fig. 2A**). The most recent serum alkaline phosphatase activity was above the upper end of normal (155 IU/L; reference range: 30-120), the $1,25(OH)_2$ vitamin D level was at the lower end of normal (19.2 pg/mL), and the 25 vitamin D level was well below the recommended range (6.4 ng/mL). His most recent urinary calcium/creatinine ratio was 0.03, while his renal function was significantly impaired. Nephrocalcinosis had been known since early childhood and current imaging by computed tomography revealed marked bilateral renal calcifications with staghorn calculi (**Fig. 2B**).

Cell culture and in vitro studies

Characterization of wild-type and mutant PTH/PTHrP receptors

GS22A cells, an HEK293-derived cell line that stably expresses the luciferase-based pGlosensor-22F (Glosensor) cAMP reporter plasmid (22, 23) were cultured at 37℃ in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. Cells were seeded in 96-well plates at a density of 2×10^4 cells per well. The following day, transfections were performed with varying amounts of each plasmid DNA (pcDNA3.1 empty vector, wild-type human PTHR1, or one of the five JMC mutants; H223R, I458K, I458R, T410P, or T410R) using FuGENE® HD Transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Assessment of receptor

expression using an antibody that specifically recognizes the human PTHR1 (rabbit polyclonal anti-hPTHR1-E2 antibody, PRB640P, LN#14861902, Covance, MA, USA) and goat anti-rabbit IgG(H+L) antibody (HRP conjugate, Prod#31460, Lot# RJ242536, Invitrogen, Carlsbad, CA, USA) was performed with enzyme-linked immunosorbent assay. Basal level of cAMP accumulation and ligand effects on PTHR1-mediated cAMP signaling were assessed 48h after transfection via the Glosensor cAMP reporter (Promega, Madison, WI, USA). Confluent cells in 96-well plates were loaded with luciferin (0.5 mM) for 25 minutes at room temperature. Subsequently, varying concentrations of agonist peptides or vehicle were added and incubations were continued for an additional period of up to 90 minutes. Luminescence arising in response to intracellular cAMP binding to the Glosensor reporter enzyme was measured at 2-minute intervals during both the pretreatment and ligand-addition phases using a PerkinElmer Envision plate reader. The area under the curve (AUC) of the luminescence response during a 25 minutes pre-ligand phase (basal) and during a subsequent 90 minutes ligand treatment phase was calculated to determine cAMP generation in cells expressing mutant or wild-type PTHR1 and to establish agonist dose-response curves. For ligand treatment experiment, vehicle or PTH(1-34) at varying concentrations (from 1×10^{-7} to 1×10^{-11} M) were added to GS22A cells transfected with 100 ng of each plasmid DNA. Aggregate data of the AUC of the luminescence response are expressed as mean ± SEM of 5 experiments, each performed in duplicate. For the inverse agonist experiment, vehicle or [L^{11},dW^{12},W^{23},Y^{36}]PTHrP(7-36) (1×10^{-6} M) were added to GS22A cells transfected with 100 ng of each plasmid DNA. The decrease in the ratio from the start point (time 0) of each luminescence response was calculated. Aggregate data are expressed as mean±SEM of 2 experiments, each performed in quadruplicate. Data were processed using Excel for Mac (Microsoft Corp) and Prism 7.0 (GraphPad Software, Inc). Curves were fit to the data using a 4-parameter, nonlinear regression function.

RESULTS

The H223R mutation was identified in 18 JMC patients ((5, 6, 10, 11, 13, 15, 16, 18, 19) and unpublished cases), while the T410P, I458R, and I458K mutations were each reported in a single case (6, 12, 14, 19); the T410R mutation was found in a father and his two sons (17). With the exception of H223R-4, H223R-13, H223R-14, T410R-2, and T410R-3, who inherited the allele from an affected parent, each other JMC patient was born to healthy parents; thus, the majority of JMC patients acquired the mutation *de novo*. Three JMC patients have children (n=5), all five of whom inherited the parental PTHR1 mutation; one affected female parent (H223R-12) has two affected sons (13), the other affected female parent (H223R-3) has an affected daughter (6), and the one affected male parent (T410R-1) has two affected sons (17).

Most patients were diagnosed with JMC during childhood. However, the affected male patient T410R-1, was not diagnosed until the age of 33 years when his two affected sons, both with the same PTHR1 mutation, presented with typical radiographic findings; these patients exhibit less severe clinical and biochemical abnormalities than most other JMC patients (17). Similarly, one female patient (H223R-3) was not diagnosed until the age of 37 years, when her daughter was found to have the JMC mutation following evaluation for achondroplasia (6). Another female patient (H223R-12), a 38-year-old mother with two affected sons, had been noted to have severe short stature since early childhood and abnormal radiographic findings, but was not overtly hypercalcemic (13); thus the JMC diagnosis was not considered until her two sons were confirmed to have the disease.

Laboratory measurements were obtained for eight patients during the first 1.5 months of life because of respiratory difficulties and/or skeletal abnormalities (see **Fig. 1** and **Suppl. Table 1**). When excluding patient H223R-17, who had a total calcium level of 13.7 mg/dL at the age of 5 days, most JMC patients evaluated during the neonatal period (n=7) had calcium levels that were within the normal range (9.6±0.64 mg/dL; mean±SD). During infancy and childhood (0.15-10 years), JMC patients with the H223R mutation (n=17) had significantly elevated total serum calcium levels (12.0±1.34 mg/dL; mean±SD; range: 9.3-14.8); similar degrees of hypercalcemia were observed also for cases with other PTHR1 mutations. The three patients with the T410R mutation had lower calcium levels at each measurement (**Fig. 3A**).

The average total serum calcium level for adult JMC patients (17-38 years; n=7) with the H223R mutation was 10.3±0.67 mg/dL, which is significantly lower than for children

affected by this disorder (infancy/childhood vs. adult: $p<0.005$). Thus, hypercalcemia in JMC is clearly more pronounced during infancy/childhood, with average calcium levels reaching the upper end of the normal range by adulthood (see **Fig. 3A**).

The average urinary calcium/creatinine ratio (mg/mg) was 0.90±0.45 (range: 0.32-1.40) for infants/children with the H223R mutation; the ratios for children with other JMC mutations were 0.80 (T410P), 0.45±0.09 (T410R), 0.71 (I458K), and 0.61 (I458R) (**Fig. 3B**). There was a strong correlation between serum calcium and the urinary calcium-to-creatinine ratio (**Suppl. Fig. 2**). Adults with the H223R mutation showed a lower, but still elevated urinary calcium excretion with an average calcium/creatinine ratio of 0.51±0.09 (infancy/childhood vs. adult: $p=0.25$). These data show that urinary calcium excretion remained above the normal range even after total serum calcium levels had improved. The serum phosphate concentrations were at the lower end of the age-specific normal range in both childhood and adulthood (**Fig. 4A**). Serum PTH concentrations for each of the different PTHR1 mutations were below or at the lower end of the reference range, except for case H223R-12 and the adult patients with the T410R mutation. PTH levels were not significantly different for children and adults (infancy/childhood vs. adult: $p=0.44$) (**Fig. 4B**). The serum alkaline phosphatase concentrations were above the age-specific normal range, except for one adult with the H223R mutation (H223R-17) and one of the two brothers with the T410R mutation. Few patients had measurements of serum $1,25(OH)_2$ vitamin D concentrations; these were within or slightly above the reference range (see **Suppl. Table 1**).

Twelve of 14 patients for whom follow-up ultrasound data were available demonstrated nephrocalcinosis; only two patients, H223R-1 and T410R-1, showed no evidence of renal calcifications when evaluated at the age of 3 and 33 years, respectively (17, 18). Two patients, H223R-16 and T410P, both older than 50 years, exhibited severe chronic kidney disease (see **Fig. 2A**) secondary to long-standing nephrocalcinosis or renal calculi, as well as urinary tract obstructions and recurrent pyelonephritis (14). Eight patients are known to have developed kyphoscoliosis and three patients revealed craniosynostosis. Eight patients had been treated with a bisphosphonate and thirteen patients had undergone surgical interventions for correction of long-bone deformities, progressive scoliosis, cranial vault reconstruction, or nephrolithotomy (see **Suppl. Table 1**).

The mean final adult height for patients with the H223R mutation was 127.0±6.0 cm for males (n=4) and 120.4±10.3 cm for females (n=5) (**Fig. 5A**). The mean adult height of the three male patients with T410R mutation was 157.7±6.4 cm, which is significantly taller than that of adult males with the H223R mutation ($p<0.002$); the final height of the single patient with the T410P mutation was 96 cm. The standard deviation scores (SDS) for height of the pediatric JMC patients were at least 2 Z-scores below the normal mean (**Fig. 5B**).

Long-term clinical outcomes of patients affected by Jansen's disease

Only two previous reports provided long-term follow-up of JMC patients, who are both females with either the T410P (14) or the H223R mutation (11). For patient H223R-11 additional data became available showing that CTX levels decreased during the 11 years of bisphosphonate treatment from a maximum of 0.79 ng/ml to approximately 0.2 ng/ml. After discontinuation of alendronate at the age of 31 yrs, her serum calcium level increased to 11.3-11.9 mg/dl and serum CTX increased to 0.30-0.37 ng/ml. The urinary calcium/creatinine ratio, which had been between 0.22-0.33 during the bisphosphonate treatment, increased after discontinuation of this medication to 0.44-0.53, despite increasing the dose of hydrochlorothiazide to 50 mg/d. At the age of 30 yrs, a renal CT showed stable bilateral microcalculi (up to 6 mm in size), but no nephrocalcinosis; serum creatinine levels remained between 0.4-0.5 mg/dl. Additional retrospectively collected clinical and laboratory findings for several other JMC patients are provided in **Fig. 1** and **Suppl. Table 1**.

Characterization of the PTHR1 mutants in HEK293-derived reporter cells

GS22A cells (HEK293 cells stably transfected with the glosensor cAMP reporter) were transiently transfected with increasing amounts of plasmid DNA (10, 20, 40, 80, and 160 ng/well) encoding either a mutant or the wild-type PTHR1. The PTHR1 mutants showed dose-dependent increases in basal cAMP levels that reached a plateau at 160 ng DNA/well. All mutant receptors showed agonist-independent cAMP generation; the T410R mutant revealed the lowest constitutive activity, while I458K-PTHR1 and I458R-PTHR1 generated a much higher basal cAMP level; there was no readily detectable increase in basal cAMP generation in cells expressing the wild-type PTHR1 (**Fig. 6A**). Similar to previously reported findings (6), cell surface expression of all

mutant receptors (100 ng/well), as determined by anti-PTHR1 antibody binding, was significantly reduced in comparison to the wild-type PTHR1 (date not shown). Each PTHR1 mutant mediated a cAMP response to increasing concentrations of PTH(1-34) that was reduced as compared to that mediated by the WT-PTHR1, except for the I458K mutant, which exhibited an increased sensitivity to the agonist ligand (**Fig. 6B**). Treatment of cells expressing the different PTHR1 mutants with the ligand analog, [L^{11},dW^{12},W^{23},Y36]PTHrP(7-36) (10^{-6} M) resulted a rapid and persistent reduction in basal cAMP signaling, consistent with the notion that this N-terminally truncated antagonist peptide can function as an inverse agonist and thus cause a decrease in the proportion of mutant receptors that are in the active-state conformation (**Fig. 6C**).

DISCUSSION

We report on clinical and laboratory observations for 24 JMC patients with information collected from shortly after birth up to the age of 56 years; serial measurements are presented for several cases. Our goal was to help assess the natural history profile for JMC, an ultra-rare, high-impact disease. We found that all but one patient had blood calcium levels that were within the reference range during the first 1.5 months of life, indicating that the development of hypercalcemia depends largely on post-natal mechanisms, which could include enhanced $1,25(OH)_2$ vitamin D-dependent intestinal calcium absorption and enhanced resorption of mineralized bone. Hypercalcemia was variable, but typically became pronounced during infancy/childhood and improved significantly by adulthood; ionized calcium was normal in the few adult cases in whom it was measured. Importantly, however, hypercalciuria with suppressed PTH secretion persisted into adulthood and likely contributed to the progressive decline in renal function that was encountered in the two older patients. In contrast, serum phosphate levels remained at the lower end of the age-specific normal range.

We also noted considerable variability in the clinical findings among different JMC patients, even in those carrying the same PTHR1 mutation. For example, female patient H223R-12 had never shown overt abnormalities of mineral ion homeostasis, whereas her two affected children were hypercalcemic by age two (13). The reason for such variations in blood calcium levels is unknown, but could involve differences in dietary intake of calcium and/or vitamin D, or some unknown genetic modifier(s) affecting calcium homeostasis. Twelve of 14 patients, for whom results of ultrasonographic studies were available, showed nephrocalcinosis.

The T410R mutation, present in three members of one family (17), appears to cause a relatively milder form of JMC, as it was not associated with major elevations in blood calcium levels, one of the three patients had normal renal ultrasound images, and the adult heights were at or close to the 3rd percentile, despite radiographic growth plate changes typical of the disease. Consistent with the less severe clinical and biochemical abnormalities associated with the T410R mutation, *in vitro* studies showed only a low level of constitutive cAMP formation for this mutant allele (17). The findings in this family with the T410R mutation make it evident that certain PTHR1 activating mutations can cause changes in the growth plates without causing major abnormalities in mineral ion homeostasis.

The I458K mutation, which had been identified only in a single pediatric case (12), showed elevated basal activity and full responsiveness to PTH(1-34). Mineral ion abnormalities and impairment of growth revealed no obvious difference when compared to patients with other PTHR1 mutations at the same age, but it will be necessary to determine whether differences can be observed later in life.

It remains uncertain as to why hypercalcemia ameliorates with age and why hypercalciuria persists in most adult JMC patients without overt hypercalcemia. Several mechanisms most likely contribute to the blood calcium elevation observed at certain times in affected individuals, namely increased bone resorption, enhanced intestinal calcium absorption, and possibly enhanced calcium reabsorption in the distal renal tubules. With the exception of a few adult patients, serum levels of alkaline phosphatase, a marker of osteoblast activity, remained above the reference range (see **Suppl. Table 1).** It is therefore conceivable that increased bone turnover with increased bone resorption persists during adulthood. Few published reports discuss the possibility of impaired renal calcium handling in JMC. In fact, only Parfitt et al. investigated the relationship between fractional calcium excretion and serum calcium levels in the JMC patient with the T410P mutation, and the authors had shown normalization of tubular calcium reabsorption with age (14). However, when the studies were performed, the patient already had significantly impaired renal function, which may have contributed to the decline in calcium excretion. Nonetheless, it appears possible that decreased serum $1,25(OH)_2$ vitamin D concentrations during adulthood, combined with reduced expression of the PTHR1 mutant in distal renal tubules and thus reduced constitutive calcium reabsorption, leads to amelioration of hypercalcemia, albeit with enhanced bone resorption and urinary calcium excretion persisting.

PTH levels in older patients remained suppressed at or below the lower limit of the reference range despite improved serum calcium levels. Circulating PTH levels are regulated mainly by the concentration of blood ionized calcium, which activates the calcium-sensing receptors expressed on the surface of parathyroid cells to thereby reduce hormone secretion (24). Although blood ionized calcium levels were available only for three adult patients (H223R-4: 1.28 (nl: 1.08-1.34) (6); H223R-11: 1.43 (nl: 1.15-1.33) (11); H223R-12: 1.25 (nl: 1.14-1.29) (13)), the measurements were above, or at the upper end of the normal range. Hence, ionized calcium may be elevated intermittently, thus

activating the calcium-sensing receptor on the parathyroid cells sufficiently to reduce PTH secretion. Importantly, low or low-normal PTH levels prevent activation of PTHR1 expressed from the normal allele, thus limiting most likely distal tubular calcium reabsorption and contributing to the hypercalciuria and nephrocalcinosis. Consequently, a decreased blood PTH level combined with an increased urine calcium excretion and typical skeletal findings may be a more reliable indicator of JMC than the blood calcium level alone, which has been normal in some patients of the current study.

Most JMC patients, whose ultrasonographic studies were available, revealed nephrocalcinosis early in life and two older patients developed severe chronic kidney disease. These complications of the disease are probably caused or accelerated by a tendency towards hypercalcemia combined with markedly increased urinary calcium and phosphate excretion. In the patient with the T410P mutation, nephrocalcinosis contributed to the chronic urinary tract obstructions, making her prone to infections (14). It is therefore important to routinely monitor renal function in adult JMC patients, as it appears to decline considerably with age, especially with recurrent pyelonephritis or obstructive uropathy.

To slow or prevent deterioration of kidney function, treatment with a bisphosphonate and the subsequent addition of a thiazide diuretic has been reported to normalize blood calcium levels and to markedly reduce urine calcium excretion in JMC patients (11, 19). Onuchi et al. documented in one patient, H223R-11, that the combination of alendronate (10 mg/d), initiated at 20 years of age, and hydrochlorothiazide initiated at 26 years of age (initially 12.5 mg/d, subsequently increased to 25 mg/d), normalized urinary calcium excretion (11). Discontinuation of alendronate at the age of 31 years led to an increase in serum and urine calcium, despite treatment with a higher dose of hydrochlorothiazide (50 mg/d), but her renal function has thus far remained stable. Although long-term outcome data for five additional patients with the H223R mutation, who had been treated with a bisphosphonate, are not yet available, it appears plausible that limiting urinary calcium excretion will help preserve renal function.

Although JMC is very rare, the impact of the disease on patient quality of life and the associated long-term health-care burden emphasize the need for an effective form of therapy. No specific treatment for JMC is currently available, however. Amino-terminally truncated PTH and PTHrP analogs with the Gly12—>dTrp substitution, originally developed as PTH antagonists (25), function *in vitro* as inverse agonists on the

constitutively active PTHR1 mutants of JMC (26, 27) (see **Fig. 6B**) and also in a transgenic mouse model of JMC (28). Whether such an inverse agonist ligand could be developed so as to suppress the elevated signaling activity of the mutant PTHR1 in bone cells, growth plate chondrocytes, and kidney cells of JMC patients remains to be investigated.

In conclusion, findings in 24 patients with JMC reveal that the final adult height of most patients is markedly reduced; only individuals with the T410R mutation, a PTHR1 mutation with only limited constitutive activity when tested *in vitro,* showed better growth. Hypercalcemia in JMC varies with age and depends at least to some extent on the intrinsic signaling properties of the specific PTHR1 mutant. Hypercalcemia improves with age, but most patients continue to exhibit long-standing hypercalciuria and thus nephrocalcinosis, which likely contributes to progressively impaired renal function. Findings *in vitro* suggest that PTHR1 inverse agonist ligands are worth exploring as a potential means of therapy for JMC.

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LEGENDS TO FIGURES AND TABLE

Fig. 1: Serum calcium concentrations of multiple patients with different PTHR1 mutations from the newborn period until the sixth decade of life; eight patients with measurements within the first 1.5 months of life are indicated at the left of the axis break. Patients are depicted by open or closed symbols of different colors, and identify these individuals in Suppl. Table 1. Patients with the H223R mutation are represented by open or closed circles; black filled circles represent patients for whom only one measurement was available; colored open or closed circles represent patients for whom multiple measurements were available. Consecutive measurements for patient H223R-17 are depicted with red circles/line. Data for three patients with the T410R-PTHR1 mutation at different ages (father, black triangle; and his two sons; blue and red triangles, respectively); measurements for the patient with the T410P-PTHR1 mutation (diamonds), I458K-PTHR1 mutation (trapezoids), and I458R-PTHR1 mutation (pentagons). Dashed lines represent the upper/lower end of the adult normal range for total calcium levels (8.6-10.2 mg/dL). The reference range for infants is 8.4-10.6 mg/dL.

Fig. 2:

Panel A: Glomerular filtration rates (GFR) as calculated by the Schwartz formula are presented for eight adult patients with three different PTHR1 mutations. For the patient with the T410P mutation (diamonds), three measurements are shown that were obtained during adulthood prior to hemodialysis that was initiated at age 37 years. For patient H223R-16 (filled circles) numerous measurements were performed after the age of 38 years showing the progressive decline in renal function.

Panel B: Latest abdominal computed tomography of patient H223R-16 at age 55 years showing extensive renal calcifications.

Fig. 3: Serum and urinary calcium measurements for multiple children (0.15 to 10 years; n=22 for serum calcium, n=15 for urinary calcium/creatinine) and multiple adults (17 to 38 years; n=11 for serum calcium; n=8 for urinary calcium/creatinine) with Jansen's disease due to different PTHR1 mutations. Each data point represents the mean, if a patient had multiple measurements during the two observation periods.

Panel A: total calcium levels; dashed lines represent the upper/lower end of the adult

normal range (8.6-10.2 mg/dL). **Panel B:** urinary calcium-to-creatinine (Ca/Cr) ratio; all individual data points are shown. Mean±SD are for patients with the H223R mutation. Dashed lines represent the upper end of normal for adult patients (<0.2). Children and adults showed no significant difference in the urinary Ca/Cr ratio.

Fig. 4: Serum phosphate levels and PTH levels at different ages for multiple patients affected by Jansen's disease due to different PTHR1 mutations. The means are shown if patients had multiple measurements during the two observation periods.
Panel A: Phosphate levels for infants (<1 year), children between 1-12 years of age, and patients older than 15 years). The lower limits of the age-dependent reference ranges for phosphate are: 0-6 months, 1.8 mmol/L (5.6 mg/dL); 6-12 months, 1.6 mmol/L (4.9 mg/dL); 1-10 years, 1.2 mmol/L (3.8 mg/dL); and >15 years, 0.8 mmol/L (2.5 mg/dL). Individual data points are shown. Mean±SD are for patients with the H223R mutation.
Panel B: PTH levels for children (0.15-10 years) and adults (17-38 years). Lower end of the adult reference range, 10 pg/ml (dashed line). Individual data points and mean±SD for patients with the H223R mutation are shown. Serum PTH levels were not significantly different for affected children and adults.

Fig. 5: Height data for different patients affected by Jansen's disease due to different PTHR1 mutations.
Panel A: Individual final heights for thirteen adult JMC patients. Mean±SD are shown for the final heights of patients with the H223R mutation; the red broken lines indicates the 3rd percentile for normal adult heights.
Panel B: Individual height Z-scores for eight children.

Fig. 6: Functional evaluation of the wild-type and different PTHR1 mutants in HEK-293/Glosensor (GS22A) cells. For some data points, the error bars are small and thus within the height of the symbol.
Panel A: The basal cAMP production in GS22A cells that were transiently transfected with increasing amounts of plasmid DNA (10, 20, 40, 80, and 160 ng/well) encoding either a mutant or the wild-type PTHR1.
Panel B: PTH-stimulated cAMP accumulation in cells transfected with plasmid DNA (100 ng/well) encoding either wild-type or mutant PTHR1s. Data are shown as the AUC

of cAMP accumulation; mean±SEM.

Panel C: Functional evaluation of the inverse agonist [L^{11},dW^{12},W^{23},Y36]PTHrP(7-36) in GS22A cells expressing the wild-type PTHR1 or different JMC mutants. The cAMP-dependent luminescence responses in cells transfected with plasmid DNA (100 ng/well) encoding either wild-type or mutant receptor. Data are shown as the AUC of cAMP-dependent luminescence measured over time after addition (t=0) of either buffer (open symbols) or inverse agonist (filled symbols); all data corrected for time 0; mean±SEM.

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Figure 1

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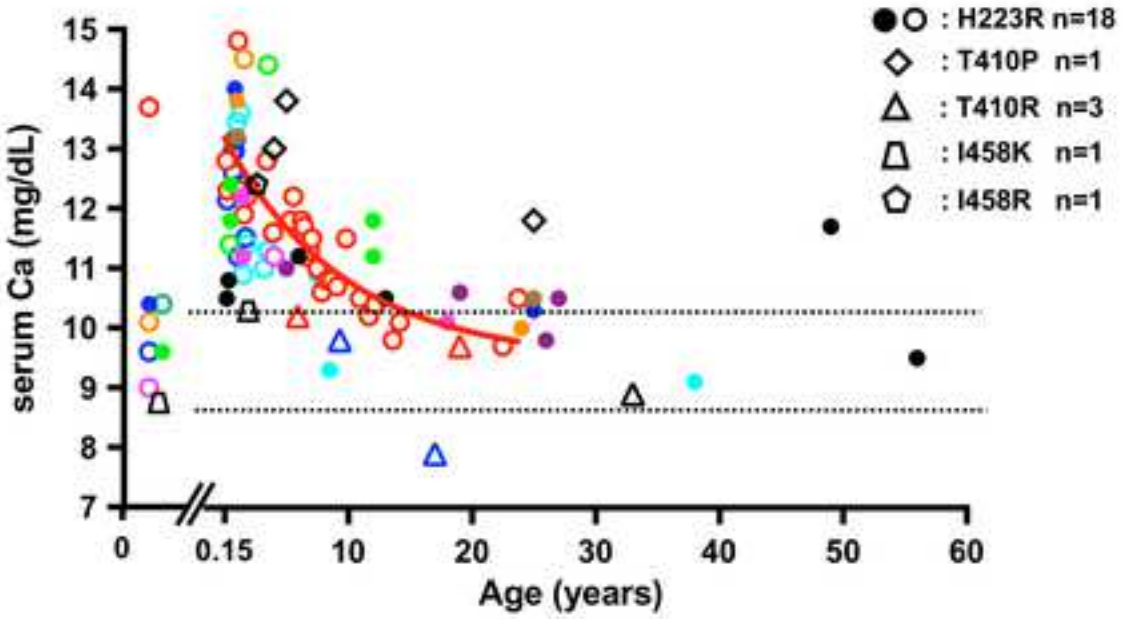

Fig.1

Figure 2

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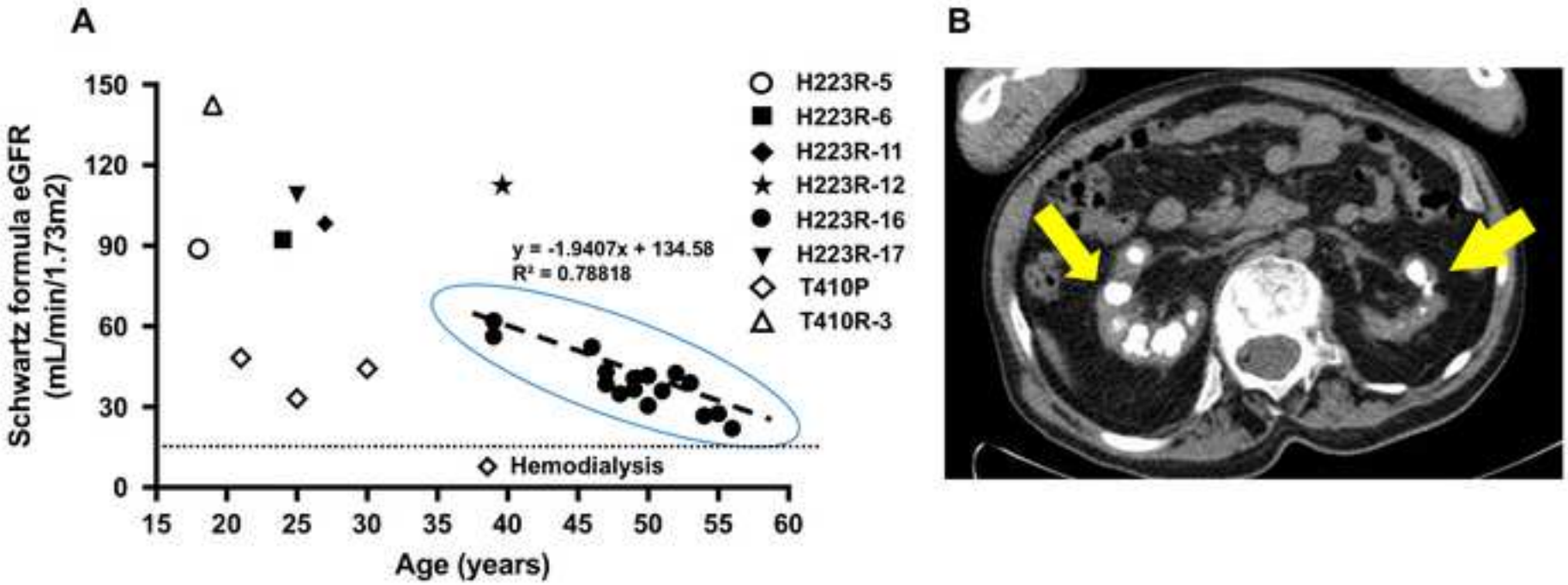

Fig.2A,B

Figure 3

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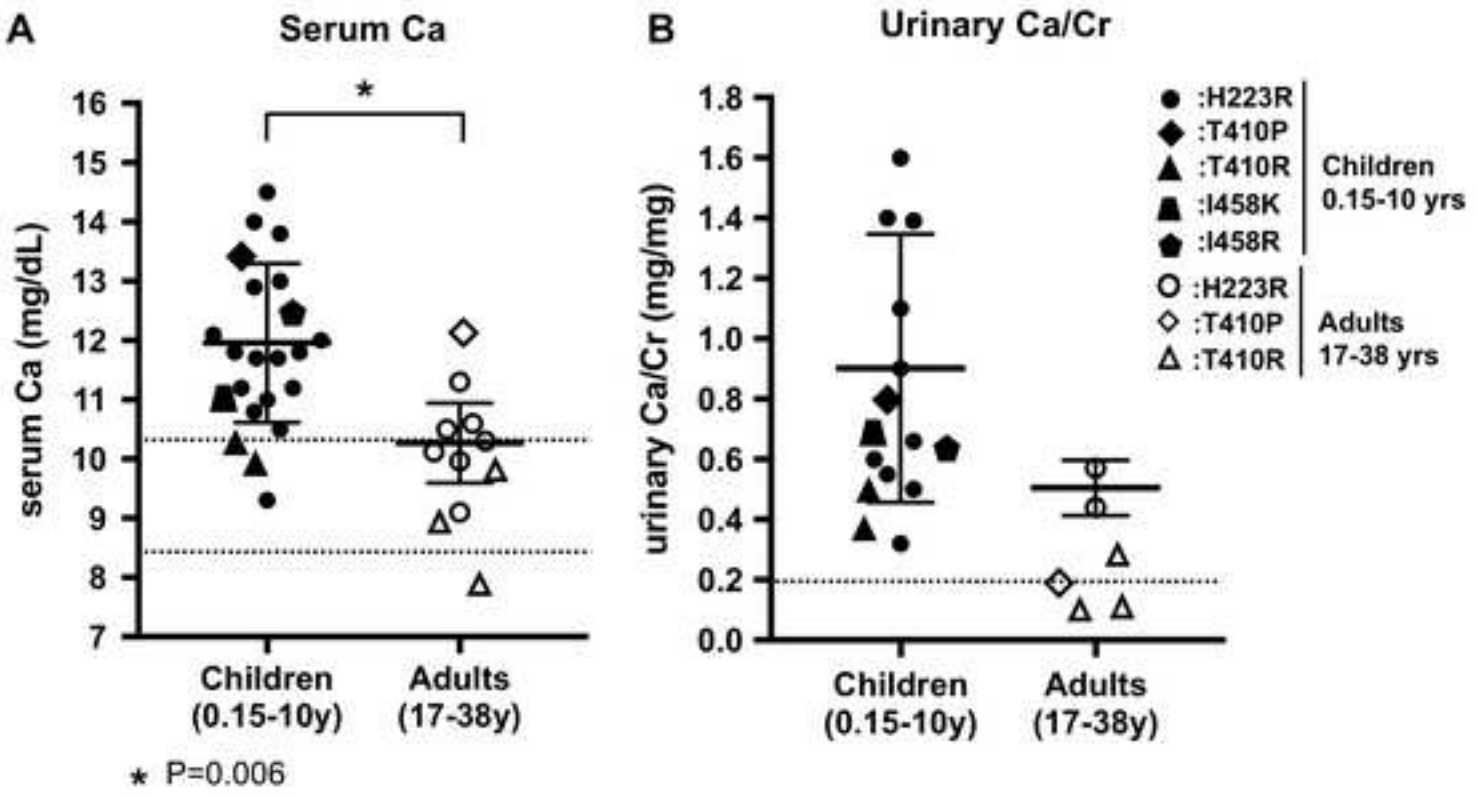

Fig.3A,B

Figure 4

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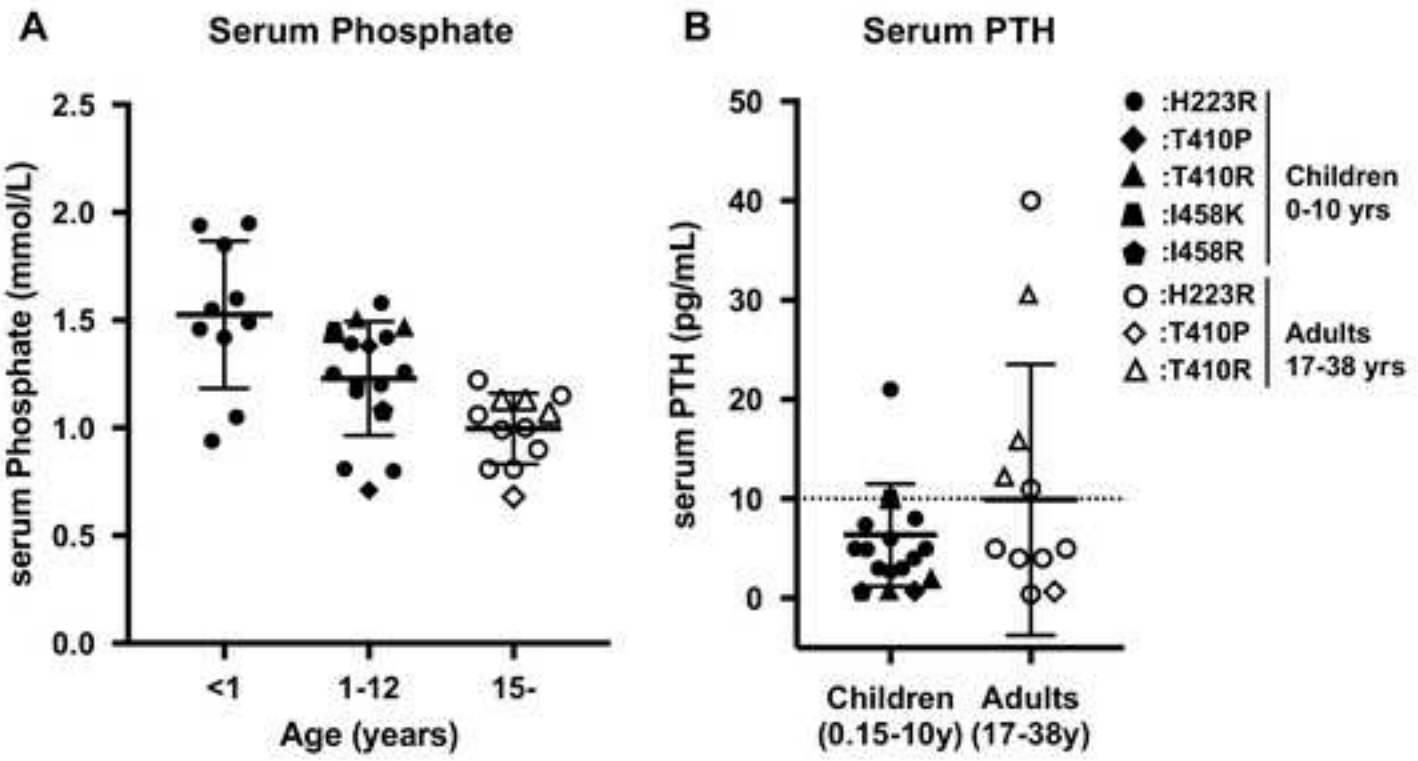

Fig.4A,B

Figure 5

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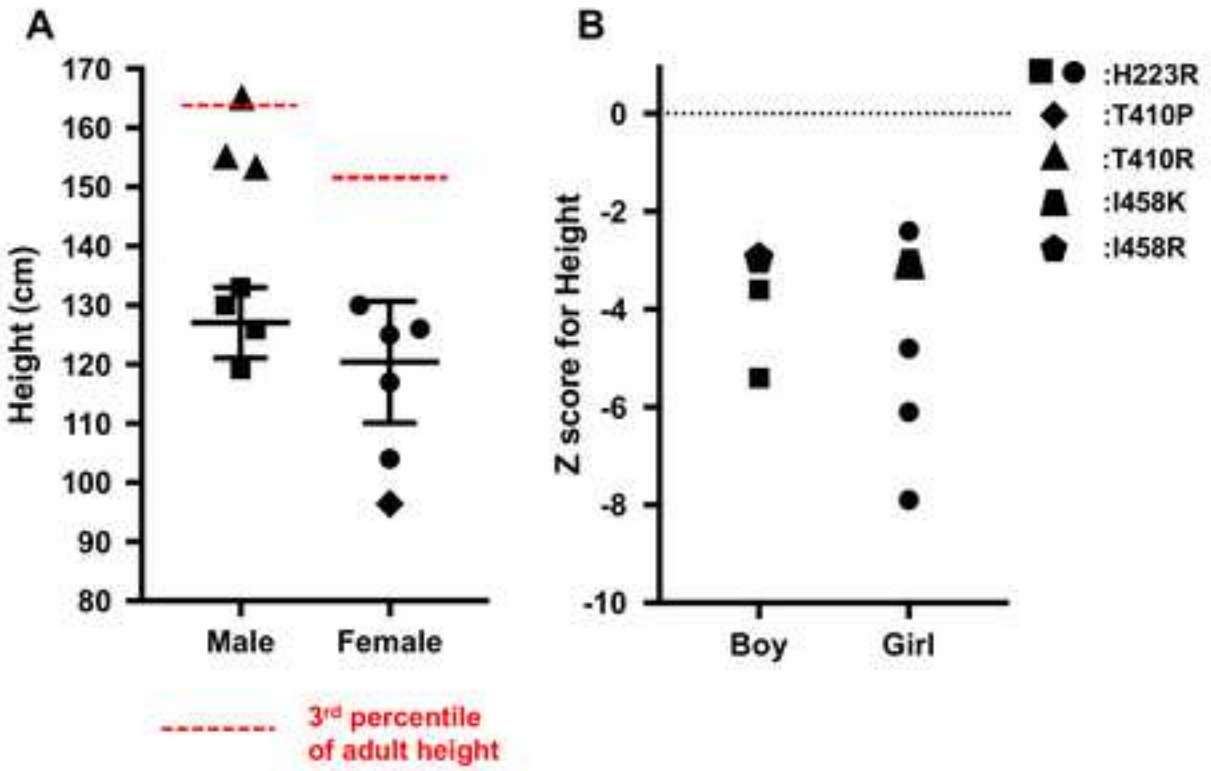

Fig.5A,B

Figure 6

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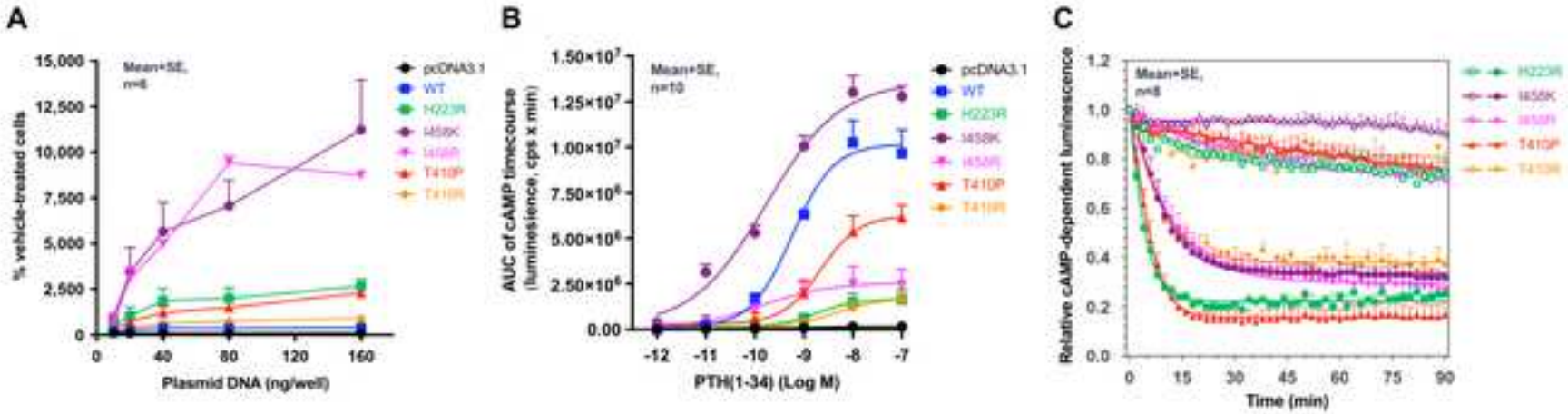

Fig.6A,B,C